Characterization of fungus-specific human T helper cell responses

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Contents

Abbreviations

α/β T cell	T cell expressing a TCR composed of Vα- and Vβ-chains
aa	amino acid
Ab	antibody
ABPA	allergic bronchopulmonary aspergillosis
AdV	adenovirus
APC	antigen presenting cell
ARTE	antigen-reactive T cell enrichment
BAL	bronchoalveolar lavage
CCR	CC chemokine receptor
CD	cluster of differentiation (only with numbers)
CD	Crohn´s Disease
CD40L	CD40 ligand
CF	cystic fibrosis
CFSE	carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptor
CMV	cytomegalovirus
CRTAM	cytotoxic and regulatory T cell molecule
CRTh2	chemoattractant receptor-homologous molecule expressed on Th2 cells
CT	computed tomography
CTL	cytotoxic T cell
CV	coefficient of variation
CXCR	CXC chemokine receptor
DC	dendritic cell
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Foxp3	forkhead box protein P3
FSC	forward scatter
γδ T cell	T cell expressing a TCR composed of Vγ- and Vδ-chains
GAD	glutamic acid decarboxylase
GALT	gut associated lymphoid tissue
GM-CSF	granulocyte-macrophage CSF
h	human
HIV	human immunodeficiency virus
HLA	human histocompatibility leukocyte antigen
HSCT	hematopoietic stem cell transplantation

IA	invasive aspergillosis
IFI	invasive fungal infections
IFN	interferon (*e.g.* IFN-γ)
Ig	immunoglobulin
IL	interleukin (*e.g.* IL-2)
KLH	keyhole limpet hemocyanin
MACS	magnetic cell separation
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MP65	*C. albicans* mannoprotein 65
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PMA	phorbol myristate acetate
r	recombinant
ROC	receiver-operating characteristic
rpm	revolutions per minute
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
pp	peptide pool
PRR	pattern recognition receptor
pTreg	peripheral regulatory T cell
SD	standard deviation
SEB	Staphylococcal enterotoxin B
SEM	standard error of the mean
SSC	side scatter
T-bet	Th1-specific T-box 1 transcription factor
Tcon	conventional T cell
TCR	T cell receptor
TGF-β	transforming growth factor β
Th cell	T helper cell
TLR	toll-like receptor
Tmem	conventional memory T cell
TNF-α	tumor necrosis factor α
Treg	regulatory T cell
Tresp	responder T cell
TSDR	Treg specific demethylated region
tTreg	thymic regulatory T cell
WBC	white blood cell
WT-1	Wilms tumor antigen

1 Introduction

1.1 T cells

T cells are the central organizers of adaptive immune responses against various pathogens and are essential in the mediation of tolerance to auto-antigens or usually harmless environmental antigens, such as commensals, allergens or food. They differentiate in the thymus from incoming bone-marrow derived lymphocyte progenitor cells into naive T cells, expressing T cell receptors (TCRs) with a single specificity that enable the recognition of antigenic epitopes. The majority of T cells (90-95%) express TCRs composed of a heterodimer of α/β immunoglobulin chains that are generated from variable, diversity, joining and constant gene fragments through somatic gene arrangement (Davis and Bjorkman, 1988). This process allows the generation of a highly diverse TCR repertoire that enables the immune system to respond to a multitude of different antigens. The theoretical number of different α/β TCRs that can be generated has been estimated to be $>10^{15}$ in mice and $>10^{18}$ in humans (Davis, 1990; Davis and Bjorkman, 1988). However direct assessment of TCR clonotypes has revealed a repertoire diversity of about 10^6 in mice (Casrouge et al., 2000) and 10^8 in humans (Arstila et al., 1999; Robins et al., 2009).

The α/β TCR recognizes peptides presented on major histocompatibility complex (MHC) molecules. During T cell development, each T cell has to pass through a process of positive and negative selection that ensures survival of those T cells, that express a TCR able to recognize self-MHC, but with a low affinity for self-peptides. Thus, each T cell in the naive repertoire that survived these selection processes should be capable to recognize self-MHC with a foreign peptide, but avoid self-peptide recognition (Hogquist et al., 2005; Jenkins et al., 2010). In general, two major subsets of α/β T cells can be distinguished. T cells expressing the co-receptor CD8 (cytotoxic cells, CTL) recognize MHC class I (MHC I) molecules, which are expressed on almost all cell types and present peptides from endogenous proteins, *e.g.* from intracellular pathogens but also self-peptides. This mechanism allows the immune system to look inside the cells and clear virus infected or tumor cells. Recognition of peptide-MHC I by $CD8^+$ T cells induces usually apoptosis of the presenting cell by release of cytotoxic molecules like perforins and granzymes or via cell surface molecules (Harty et al.,

2000; Trapani and Smyth, 2002; Van Parijs and Abbas, 1996). In contrast, T cells expressing the co-receptor CD4 (T helper cells, Th) recognize MHC class II (MHC II) molecules. MHC II is expressed on professional antigen-presenting cells (APCs), such as dendritic cells (DCs), B cells or macrophages that present peptides from exogenous proteins, *e.g.* from extracellular pathogens, but also normally harmless antigens like allergens, food, *etc.* Activated $CD4^+$ T cells conduct a variety of different functions: they provide help to B cells to produce antibodies, support priming and maintain responses of $CD8^+$ T cells, control recruitment and migration of innate immune cells and also regulate/ suppress immune responses to control autoimmunity and to dampen overwhelming immune reactions (Swain et al., 2012; Zhu et al., 2010).

1.1.1 Functional heterogeneity of $CD4^+$ T cells

Upon antigen encounter in the secondary lymphoid organs, those naive T cells that express a TCR specific for the antigen undergo clonal expansion and differentiate into effector cells that orchestrate specific immune responses. The differentiation into effector cells is accompanied by the acquisition of distinct effector functions that enable an adequate and protective immune reaction depending *e.g.* on the nature of the pathogen and/ or the site of exposure. Following clearance of the antigen, the expanded pool of effector T cells contracts, leaving a smaller population of persistent memory cells, which maintain the acquired effector functions and provide protection upon re-exposure to the antigen (Jenkins et al., 2001).

The kind of effector response is mainly determined by the cytokine milieu during the first antigen encounter, although other factors like the affinity to the TCR might play a role (Zhu and Paul, 2010). Polarizing cytokines produced by APCs induce the expression of master transcription factors, which in turn determine the cytokine producing capacity of the T cell (Murphy and Reiner, 2002). In particular $CD4^+$ T cells are heterogeneous and conduct a wide range of different effector functions, which allow their further classification into several Th cell subsets. In addition to their cytokine-producing capacities, Th cell subsets can be classified according to the expression of several homing receptors that are induced during polarization and mediate selective recruitment of the T cells to the site of antigen uptake (Bromley et al., 2008; Butcher and Picker, 1996; Sallusto et al., 2004).

Th1 cells differentiate in presence of IL-12 and IFN-γ, express the transcription factor T-bet (Rogge et al., 1997; Szabo et al., 2000) and the chemokine receptors CXCR3 and CCR5

(Sallusto et al., 1998). They are characterized by production of the pro-inflammatory cytokines IFN-γ and TNF-α, which activate macrophages and other immune cells. In addition, Th1 cells produce IL-2, a key cytokine for T cell proliferation and homeostasis. In contrast, Th2 differentiation occurs in presence of IL-4 and requires the transcription factor GATA-3 (Zheng and Flavell, 1997). Th2 cells express the homing receptors CCR3, CCR4 and CRTh2 and the cytokines IL-4, IL-5, IL-13, IL-25 and are important in providing help for antibody class switching and recruitment of eosinophils. Th17 cells express the transcription factor Ror-γt (Ivanov et al., 2006) and have been shown to differentiate in presence of IL-6, IL-23, IL-1β and/ or TGF-β, although the requirements seem to differ between mouse and human Th17 cells and the role of TGF-β in human Th17 cell differentiation is still a matter of debate (Acosta-Rodriguez et al., 2007a; Cosmi et al., 2008; Das et al., 2009; Manel et al., 2008; Santarlasci et al., 2009; Volpe et al., 2008; Yang et al., 2008). In addition, the differentiation requirements may vary depending on the nature of the pathogen (Zielinski et al., 2012). Th17 cells are further characterized by the expression of the chemokine receptors CCR6 and CCR4 (Acosta-Rodriguez et al., 2007b; Annunziato et al., 2007) and production of the cytokines IL-17, IL-21 and IL-22 (Liang et al., 2006). IL-17 induces recruitment and activation of neutrophils and triggers the secretion of pro-inflammatory cytokines by a variety of different immune cells (Harrington et al., 2005). In contrast, IL-22 has been shown to act mainly on epithelial cells and triggers the expression of anti-microbial peptides (Ouyang and Valdez, 2008; Wolk et al., 2004). More recently, Th22 cells have been introduced as another potential Th subset that is characterized by secretion of IL-22, but neither IL-17 nor IFN-γ. Th22 cells express in addition to CCR6 and CCR4 the chemokine receptor CCR10, which facilitates migration into the skin and may therefore play an important role in skin immune homeostasis and pathology (Duhen et al., 2009; Trifari et al., 2009).

Another subset of $CD4^+$ T cells are regulatory T cells (Treg). They are important suppressors of immune reactions and are crucial for the maintenance of peripheral tolerance by their ability to suppress autoimmunity, immunopathology and tissue destruction (Sakaguchi et al., 2010). Critical for the suppressive capacity of Treg, is the transcription factor Foxp3, which controls the expression of genes essential for Treg homeostasis and function (Fontenot et al., 2003; Hori et al., 2003). Furthermore, Treg are characterized by their constitutive expression of CD25, the IL-2 receptor α-chain. $Foxp3^+$ Treg have been shown to arise in the thymus during $CD4^+$ T cell development, maybe from those precursors with an affinity for self-peptide-MHC at the upper end of the positive selection range (thymic regulatory T cells, tTreg) (Jenkins et al., 2010; Josefowicz and Rudensky, 2009). However, they can also be

induced in the periphery from naive $CD4^+$ T cells (peripheral Treg, pTreg), following encounter with specific antigens (Bluestone and Abbas, 2003). The factors that determine Treg differentiation in the thymus as well as in the periphery are not completely understood, but several evidences suggest, that the affinity and strength of TCR binding and antigen presentation might play an important role (Apostolou and von Boehmer, 2004; Bensinger et al., 2001; Gottschalk et al., 2010; Graca et al., 2005; Kretschmer et al., 2005).

Previous research has established a connection between the pattern of the Th cell response and the nature of the pathogen or antigen encountered. For example Th1 cells have been shown to mediate immune responses against intracellular viral and bacterial antigens. In contrast, Th2 cells mediate host defense against extracellular parasites like helminths, but are also involved in promotion of allergy to normally harmless environmental antigens, *e.g.* pollen, house dust mite or food. Th17 cells have been implicated to play a major role in immune responses at mucosal surfaces and are believed to be critical in host defense against bacteria and fungi. However, pathogenic Th17 responses are also strongly linked to several autoimmune diseases and tissue destruction (Zhu and Paul, 2008; Zhu and Paul, 2010). Finally, several evidences from mouse models suggest, that Treg and in particular pTreg play an important role in maintaining tolerance against commensals or inhaled antigens encountered at mucosal sites (Bilate and Lafaille, 2012; Curotto de Lafaille et al., 2008; Curotto de Lafaille and Lafaille, 2009; Josefowicz et al., 2012).
Although the classification of Th cell subsets in general, and establishments of the relation between pathogens and the class of Th cell response are useful for a better understanding and dissection of memory T cell responses, they might oversimplify the complex heterogeneity of T cell responses against a certain antigen. Especially for complex pathogens it is likely that immune responses are mediated by several Th cell subsets that all together contribute to protective immunity. In addition, there is a lack of knowledge about the antigen specificity of pathogen-reactive Th cells, in particular of regulatory T cells, as well as their frequency and phenotype within the human T cell repertoire, or their relation to the conventional T cell response specific for the same antigen. Therefore more detailed analyses of antigen-specific T cell responses need to be obtained that provide comprehensive information about antigen specific naive, memory as well as Treg within the human T cell repertoire. This will help to understand the mechanisms of protective immunity *versus* disease progression or immune pathology.

1.2 Antigen-specific T cells

T cells are the central organizers of adaptive immune responses. Within the total T cell repertoire, however, only the very small number of cells with specific T cell receptors enables the specific defense against a certain antigen. Information about the frequency, phenotype and function of those antigen-specific T cells is essential to gain information about the immune status of an individual and to understand the mechanisms of protective immunity in healthy donors *versus* disease progression in different patient groups or the development of immunopathology, *e.g.* autoimmunity or allergy.

The major challenge for the analysis of T cells specific for a certain antigen is their low frequency within the vast pool of T cells with irrelevant specificities. In highly boosted immune responses, such as acute infections, persistent viruses or immediately after vaccination, transient frequencies of several percent can be detected within the total T cell compartment. However, in absence of acute infections, the frequencies of specific T cells are usually only in the range of 0.01-1%. This also applies to the memory T cell pool, where the specific T cells have already undergone clonal expansion. For rare cells, such as auto-antigen- or allergen-specific T cells, the frequencies are even lower (1 cell per 10^4-10^5) and finally within the naive repertoire, T cells specific for a single peptide-MHC can be found with frequencies of only 1 cell per 10^5-10^7 naive T cells (Alanio et al., 2010; Geiger et al., 2009; Jenkins et al., 2010; Jenkins and Moon, 2012; Kwok et al., 2012).

These low frequencies of antigen-specific T cells make it obvious that methods for their analysis need to be highly specific, to detect only those cells that are specific for the antigen, and furthermore need to enable the processing of large cell numbers to collect sufficient numbers of target cells for statistically relevant analyses. Due to these high technical requirements, antigen-specific T cells could for long time be measured only indirectly following *in vitro* expansion. However, *in vitro* expansion of antigen-specific T cells can induce an unpredictable bias to the cells in terms of phenotypical and functional modifications, outgrowth of certain T cell clones or proliferation due to bystander activation. This makes it impossible to draw conclusions from *in vitro* expanded T cells on their *ex vivo* properties and to determine the actual frequency of cells that are specific for a certain antigen. Therefore, the direct *ex vivo* detection and characterization of specific T cells has become an ultimate goal for an unbiased knowledge about antigen-specific T cell responses.

In the recent years, several flow-cytometric methods for the *ex vivo* identification of antigen-specific T cells have been developed (Scheffold and Kern, 2000; Thiel et al., 2004). These

methods enable either the direct identification of the specific T cells *via* binding of the TCR to its MHC-peptide ligand (Altman et al., 1996), or their identification following activation with the specific antigen and measurement of functional parameters, *e.g.* cytokine production or the expression of activation markers (Scheffold and Kern, 2000; Thiel et al., 2004). The combination of these technologies with magnetic enrichment approaches allows the pre-enrichment of the antigen-specific T cells from large cell numbers, which drastically increases sensitivity and enables the analysis of rare antigen-specific T cells even in the naive repertoire. So far, the magnetic enrichment of cytokine producing T cells (Brosterhus et al., 1999), as well as MHC multimer labeled cells (Day et al., 2003; Moon et al., 2007) has successfully been used for the analysis of rare antigen-specific T cells undetectable via conventional methods. However, especially for complex pathogens, containing hundreds or thousands of different target epitopes, several limitations still restrict a comprehensive and unbiased insight into the heterogeneous T cell response. Thus, a method for the direct *ex vivo* analysis of $CD4^+$ T cells specific for an unrestricted pool of T cell epitopes from any antigen of interest at high resolution is still missing. Such a method should be sensitive enough to enable the analysis of the total antigen-specific $CD4^+$ compartment, *i.e.* memory, naive, as well as regulatory T cells, whose target antigens remain so far largely elusive.
The development of a method that fulfills these requirements was part of this dissertation (Bacher et al., 2013; manuscript 3; Bacher et al., 2013; manuscript 2). The different approaches for the detection of antigen-specific T cells and their limitations are reviewed in detail in chapter 3.1 of this thesis (Bacher and Scheffold, 2013; manuscript 1).

1.3 Human fungal diseases

Ubiquitous fungi are among the most common microbes encountered by humans (Wüthrich et al., 2012). It is estimated that everybody routinely inhales several hundreds or thousands of fungal spores per day (Latge, 1999; Park and Mehrad, 2009). In addition, several fungi, like *Candida* species, are commensals on skin epithelia and the gut mucosal surfaces of mammalian hosts (Iliev et al., 2012). Depending on the immune status of an individual, environmental fungi can cause a wide range of human diseases: in immunocompetent individuals fungal diseases range from superficial mycosis to allergic hypersensitivity and non-invasive colonization. However, in immunocompromised patients fungi can even cause life-threatening invasive infections with often fatal outcome (Brakhage, 2005).

Most fungus-related allergic diseases including asthma, sinusitis and alveolitis occur following repeated exposure and consequently sensitization to fungal antigens but without fungal colonization or invasion. In contrast, the most severe allergic pulmonary complication, allergic bronchopulmonary aspergillosis (ABPA), is characterized by recurrent pulmonary infiltrates and mycelial growth in the lung (Latge, 1999). ABPA is mainly caused by *Aspergillus fumigatus* and occasionally also other *Aspergillus* species (Moss, 2005). It affects approximately 1-2% of patients with persistent asthma and 5-15% of patients with cystic fibrosis (Moss, 2002; Stevens et al., 2003). Due to compromised mucociliary clearance in these patients, *A. fumigatus* spores can colonize the bronchial airways and germinate into mycelia. Although lungs of ABPA patients are colonized with *A. fumigatus* mycelia, no invasive growth of the fungus into tissues and blood vessels is observed. The disease is characterized by a strong Th2 response against fungal antigens, leading to symptoms of severe asthma that may proceed to airway destruction, bronchiectasis and lung fibrosis (Knutsen et al., 2012; Knutsen and Slavin, 2011; Latge, 1999). An ongoing problem is the diagnosis of ABPA, since the diagnostic criteria like asthma, peripheral blood eosinophilia, increased total and fungus specific IgE levels, skin reactivity to *A. fumigatus* antigens, expectoration of mucus plugs and pulmonary infiltrates are rarely present in all patients and are furthermore not specific for the disease or overlapping with signs and symptoms of cystic fibrosis (Knutsen and Slavin, 2011; Latge, 1999). The failure to detect ABPA may lead to permanent lung damage and thus significant morbidity and mortality of the patients (Knutsen and Slavin, 2011).

Invasive fungal infections (IFI) are the most devastating disease caused by ubiquitous fungi. They occur primary in immunocompromised patients, where deficits in the host defense render them susceptible. Upon arrival in the lungs, fungal spores can germinate into hyphal forms that invade the lung parenchyma and blood vessels and may further disseminate to different organs. Due to increasing numbers of immunocompromised patients, the incidence of IFIs is rising and despite pharmacotherapeutic improvements, the mortality rate of these infections is still unacceptably high (Brown et al., 2012). The most common cohorts of patients at risk for IFIs include leukemic patients, hematopoietic stem cell or solid organ transplant recipients (De Pauw et al., 2008; Kontoyiannis et al., 2010; Low and Rotstein, 2011). Within these groups, the incidence and ethiopathology of IFIs differs depending on the underlying conditions of disease and treatment (Low and Rotstein, 2011). Among hematopoietic stem cell transplant (HSCT) recipients, a recent multicenter study covering 250 cases of IFIs from 2004 to 2007 reported, that invasive aspergillosis (59.2%) was the most

frequent, followed by invasive candidiasis (24.8%), Mucorales infections (7.2%), and IFI due to other molds (6.8%) (Neofytos et al., 2009). Although *Candida* spp. has for long time been considered as being the predominant fungal pathogen in immunosuppressed patients, there is apparently a change in the epidemiology of IFIs with rising prevalence of invasive aspergillosis (IA) as well as mucormycosis and other mold infections (Low and Rotstein, 2011; Neofytos et al., 2009). The trend towards more invasive mold infections is serious, because the mortality rate differs depending on the infecting fungal pathogen, with numbers ranging from 35.5% for IA to 64.3% for Mucorales infections and 80% for other mold infections (Neofytos et al., 2009).

A major factor in increasing the survival of infected patients is the early initiation of the appropriate antifungal therapy, which is mainly hampered by a timely and reliable diagnosis (Chamilos et al., 2008; Cornely et al., 2011; De Pauw et al., 2008; Greene et al., 2007; Mengoli et al., 2009; Ostrosky-Zeichner et al., 2005). The diagnosis of IFI is challenging, because the clinical manifestations are often not specific for fungal infections and the current diagnostic tools (*e.g.* blood cultures, detection of *Aspergillus* spp. cell wall antigen galactomannan, detection of fungal β-1,3-D-glucan, or amplification of fungal DNA by PCR) have been shown to be highly variable regarding sensitivity and specificity (Mengoli et al., 2009). In addition, the results are often available too late to be clinically useful.

Given the changing epidemiology of IFI, a further challenge for the diagnosis, is the species-specific discrimination, as rare species are becoming more common and require different anti-fungal treatment (Auberger et al., 2012; Bitar et al., 2009). Usually, only in proven IFI cases, the distinct identification of the invading fungal pathogen is possible. To diagnose a proven IFI, it requires the direct microscopic or cultural detection of the fungus in a sample taken from the infected site (De Pauw et al., 2008). Such invasive surgical procedures can rarely be applied to the majority of critically ill patients and rather represent a last therapeutic option, than a feasible diagnostic tool. Additionally, the histological examination of these samples may not be specific enough to clearly distinguish similar filamentous fungal species (Low and Rotstein, 2011; Ostrosky-Zeichner, 2012). The further classification of IFI diagnosis into probable and possible cases relies on a high value of suspicion, and requires a combination of the presence of a host factor (*e.g.* neutropenia), characteristic findings on lung computed tomography (CT) scans and microbiological findings (De Pauw et al., 2008; Low and Rotstein, 2011). Thus there is an urgent need for new diagnostic methods for the early detection of IFI with high sensitivity and specificity that allow distinct discrimination between different fungal pathogens to enable an early and targeted antifungal therapy.

1.4 Immune responses against fungal pathogens

Of the estimated 10^5 fungal species, only few hundreds cause diseases in humans (LeibundGut-Landmann et al., 2012). Two of the most commonly encountered fungal pathogens are *Aspergillus fumigatus* and *Candida albicans*. *A. fumigatus* is an ubiquitous mold, acquired primary by inhaling airborne spores, whereas *C. albicans* is a dimorphic commensal, that occurs as a yeast and hyphal morphotype and forms normal part of the human microbiota. Although all humans are routinely in contact with both fungi, the majority of people do not develop any signs or symptoms of disease. This is attributed to efficient innate and adaptive immune responses that are activated upon recognition of fungal particles and prevent fungal colonization and invasive diseases (Brakhage et al., 2010; Romani, 2011; Wüthrich et al., 2012).

The surface of resting *A. fumigatus* spores is covered by a hydrophobin layer, which masks fungal structures and avoids their immune recognition (Aimanianda et al., 2009). However, resting conidia rapidly become swollen within few hours upon arrival in the lungs as a first step of germination (Osherov and May, 2001; Park and Mehrad, 2009). This maturation process is associated with a profound rearrangement of the conidial surface leading to loss of the hydrophobin layer and exposure of several structures that are recognized by toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Brown, 2011; Hasenberg et al., 2011). Similar alterations of the cell wall structure apply for *C. albicans* during transition from yeast to hyphal form, allowing distinct recognition of both morphotypes by pattern recognition receptors (PRRs) (Hernandez-Santos and Gaffen, 2012). Upon fungal recognition, activated lung-resident alveolar macrophages and neutrophils provide first line protection against inhaled fungal spores by phagocytosis and killing of fungal particles as well as secretion of antimicrobial compounds and pro-inflammatory cytokines (Behnsen et al., 2007; Hasenberg et al., 2011). The elimination of inhaled fungal spores is highly efficient with ~ 90% of fungal spores being cleared within 24-48 hours after inoculation (Murdock et al., 2011; Philippe et al., 2003). Neutrophils have been shown to be essential in this early anti-conidial defense (Mircescu et al., 2009), as well as in killing of hyphae or capturing of fungal structures through the formation of extracellular traps (Bruns et al., 2010). Also in *C. albicans* infection, cytokines released by damaged epithelial cells recruit neutrophils that directly kill *C. albicans* cells through phagocytosis and degranulation (Naglik et al., 2011). In line with their

important role in anti-fungal immune defense, neutropenia in immune-suppressed patients is considered as a main risk factor for developing invasive fungal diseases.

Although early immune defense against *A. fumigatus* and *C. albicans* is mediated by innate immune cells, several evidences suggest, that adaptive immunity, and in particular $CD4^+$ T cells may also contribute to the immune defense against both fungi. For example, non-neutropenic patients with advanced AIDS, who lack $CD4^+$ T cells, are highly susceptible to invasive fungal infections (van de Veerdonk and Netea, 2010). Furthermore, a considerable number of invasive fungal infections in HSCT patients occur at a time point, when neutropenia has already resolved, but patients still suffer from T cell deficiency (Lehrnbecher et al., 2013). Dendritic cells and monocytes have been shown to take up fungal spores and hyphae and prime specific $CD4^+$ T cell responses *in vitro* and *in vivo* (Bozza et al., 2002; Hohl et al., 2009; Rivera et al., 2006; Rivera et al., 2005; Serbina et al., 2009). In adoptive transfer models of immune-supressed or T cell deficient mice, transfer of $CD4^+$ T cells prolonged survival after *A. fumigatus* or *C. albicans* infection (Cenci et al., 2000; Farah et al., 2002). Similarly, transferred dendritic cells pulsed with *A. fumigatus* conidia or *C. albicans* yeasts generated protective anti-fungal immunity by activation of IFN-γ producing Th1 cells (Bozza et al., 2003; d'Ostiani et al., 2000).

In general, Th1 responses mediated by IFN-γ are thought to confer protective immunity to several pathogenic fungi (Romani, 2011; Wüthrich et al., 2012), most likely by enhancing recruitment of phagocytes and fungal killing. Also in humans, a predominant IFN-γ production of *A. fumigatus* stimulated $CD4^+$ T cells has been observed in patients that survived invasive aspergillosis (Hebart et al., 2002) and adoptive transfer of IFN-γ producing T cell clones correlated with a decrease of galactomannan levels in invasive aspergillosis patients (Perruccio et al., 2005). In contrast, several studies suggest, that Th2 cytokines are detrimental in anti-fungal immune responses by counter-regulation of protective Th1 responses and promotion of allergic immune reactions in murine models and patients with invasive fungal disease and ABPA (Cenci et al., 1999; Haraguchi et al., 2010; Hernandez et al., 2005; Kreindler et al., 2010). The Th17 subset has also been implicated to play a major role in mucosal immunity against fungi. IL-17 and IL-22 produced by Th17 cells are important inducers of neutrophil recruitment and anti-microbial peptide expression from epithelial cells (Hernandez-Santos and Gaffen, 2012). Thus, Th17 cells are in general believed to have a protective role in fungal infections. This is supported by several studies with IL-17 receptor deficient mice that demonstrated an increased susceptibility to disseminated and oropharyngeal candidiasis (Conti et al., 2009; Huang et al., 2004). Also in

human patients an impaired IL-17 response directly correlates with increased susceptibility to *Candida* infections (Eyerich et al., 2008; Ma et al., 2008; Milner et al., 2008). However, the role of Th17 cells in host defense against *A. fumigatus* is less clear. Although IL-17 and IL-22 have been shown to enhance *A. fumigatus* clearance in mice (Gessner et al., 2012; Werner et al., 2009), other studies even implicate that Th17 cells may have adverse effects on anti-fungal immunity by driving pathologic inflammatory responses and impairing antifungal immune resistance in mouse models of pulmonary aspergillosis (Romani et al., 2008; Zelante et al., 2009; Zelante et al., 2007). Thus, the current data regarding the potential role of Th17 cells in immune responses against fungi are ambiguous.

Since $CD4^+$ T cells seem to play a role in anti-fungal immune responses, a potential strategy to protect from fungal infections could be the development of an anti-fungal vaccine that induces a specific and protective memory repertoire of fungus-specific $CD4^+$ T cells. In *A. fumigatus* infection models, vaccination with crude fungal lysate has been shown to protect mice from invasive aspergillosis (Cenci et al., 2000; Ito and Lyons, 2002; Liu et al., 2011). However, immunocompromised patients, who have usual low T and B cell counts, are at particular risk for developing serious invasive fungal infections, questioning the practicability of a vaccine protection in these patients. Alternatively, the restoration of specific anti-fungal immunity in immunocompromised patients via adoptive transfer of fungus-specific $CD4^+$ T cells seems to be a promising strategy (Beck et al., 2006; Gaundar et al., 2012; Perruccio et al., 2005; Tramsen et al., 2009; Tramsen et al., 2013). However, there is only fragmentary knowledge about the frequency, phenotype and function, as well as the precise antigen-specificity of fungus specific $CD4^+$ T cells in humans, which would be a prerequisite for such immuno-therapeutic approaches. Although the presence of *A. fumigatus* and *C. albicans* specific T cells has been demonstrated in humans (Acosta-Rodriguez et al., 2007b; Chai et al., 2010; Chaudhary et al., 2010; Hebart et al., 2002; Liu et al., 2009; Zhou et al., 2008), most of these analyses employed long-lasting *in vitro* culture and/or population-based methods (^{3}H-thymidine incorporation, ELISA) or ELIspot to analyze fungus-specific T cell responses. These methods suffer from prolonged *in vitro* manipulation, making it difficult to draw conclusions on the *ex vivo* phenotype of the specific T cells, or are restricted to certain cytokine producing subsets, which may represent only a fraction of all reactive T cells. In particular direct *ex vivo* analyses describing the total repertoire of human fungus-specific T cells without prolonged *in vitro* manipulation are so far missing. In addition, due to the complexity of fungal pathogens, comprehensive information about the antigens recognized by human $CD4^+$ T cells is almost completely lacking.

1.5 Aim of the study

Fungi represent a challenge for the immune system since they can occur as usually harmless commensals or daily encountered environmental antigens, but can also cause detrimental diseases as opportunistic pathogens or allergens. Antigen-specific $CD4^+$ T cells play a major role in balancing immune responses to harmless antigens and orchestrate protective immune responses against pathogens. However, their role in anti-fungal immunity is only poorly understood. In addition, the contribution of specific Treg in anti-fungal immune responses, for example for the prevention of adverse reactions, is completely unknown. The main roadblock to gain information about fungus-specific T cells is the lack of appropriate methods for their identification. Such methods should allow the direct *ex vivo* identification and characterization of rare fungus-specific T cells from human peripheral blood or tissues with high sensitivity and specificity by using an unrestricted pool of antigens, since no immune-dominant fungal T cell targets are known. Information about fungus-specific T cells will provide basic knowledge on human anti-fungal immune responses and will allow to define pathogen-specific defense mechanisms, as well as differences in anti-fungal T cell responses in relevant patient cohorts. Therefore, the aim of this study was to identify and characterize the human fungus-specific $CD4^+$ T cells in healthy donors and various patient groups, with a focus on *Aspergillus fumigatus* and *Candida albicans* as two major opportunistic fungal pathogens in humans.

The following objectives were addressed in course of this study:

1. Direct *ex vivo* detection of rare fungus-reactive $CD4^+$ T cells including Treg in human peripheral blood.
2. In depth phenotypical and functional characterization of the $CD4^+$ T cell response against *A. fumigatus* and *C. albicans*.
3. Comparison of T cell phenotype and function between healthy donors and patient groups with fungus-related pathologies.
4. Analysis of single antigen specificities of *A. fumigatus*-reactive T cells and identification of potentially immunogenic *in vivo* targets.

The detailed knowledge about human fungus-reactive T cells is essential to define correlates of protection against fungal diseases and disease progression and will help develop pre-emptive or therapeutic intervention against fungus-related pathologies.

2 Overview of manuscripts

Manuscript I

"Flow-cytometric analysis of rare antigen-specific T cells."

Bacher P and Scheffold A.

Review article published in
Cytometry A. 2013 Aug; 83(8):692-701.

Summary:
Detection of antigen-specific T cells is challenging due to the low frequencies of the specific T cells. Especially the direct *ex vivo* identification and characterization of these cells is essential to gain unbiased knowledge about antigen-specific T cell responses. In this review article, the current flow-cytometric technologies for analyses of antigen-specific $CD4^+$ T cells were summarized, with a focus on enrichment methods that provide sufficiently high sensitivity for the *ex vivo* characterization of very rare antigen-specific T cells even in the naive or regulatory T cell repertoire.

Author contribution:
Petra Bacher wrote this review article together with Alexander Scheffold.

Prof. Dr. Axel A. Brakhage

Manuscript II

"Antigen-Reactive T cell Enrichment for Direct, High-resolution Analysis of the Human Naive and Memory Th Cell Repertoire."

Bacher P, Schink C, Teutschbein J, Kniemeyer O, Assenmacher M, Brakhage AA, Scheffold A.

Manuscript published in
The Journal of Immunology, Vol. 190, pp. 3967-3976, 2013.

Summary:
$CD4^+$ T cells play a central role in the immune defense against pathogens or tumors but also in immunopathology, such as autoimmunity or allergy. Due to technical limitations, comprehensive information about the frequency and phenotype of antigen-specific $CD4^+$ T cells against many disease-relevant antigens is missing. This paper describes the development of a highly sensitive detection system for rare antigen-reactive $CD4^+$ T cells directly from human peripheral blood. Based on a pre-enrichment strategy of CD154 expressing T cells, antigen-reactive Th cells can be visualized and characterized in detail by using multi-parameter flow-cytometry. This system allows for the first time the direct *ex vivo* access and in depth characterization of specific Th cells at high resolution within the naive and memory $CD4^+$ T cell repertoire, but without any restriction to pre-selected epitopes or MHC alleles.

Author contribution:
Petra Bacher performed all experiments described in this manuscript, except of some experiments in Figure 5B that were performed by Christian Schink. Furthermore she was significantly involved in writing the manuscript.

Prof. Dr. Axel A. Brakhage

Manuscript III

"Antigen-specific Expansion of Human Regulatory T Cells as a Major Tolerance Mechanism against Mucosal Fungi."

Bacher P, Kniemeyer O, Schönbrunn A, Sawitzki B, Assenmacher M, Rietschel E, Steinbach A, Cornely OA, Brakhage AA, Thiel A, Scheffold A.

Manuscript published in
Mucosal Immunology, advanced online publication, 2013.

Summary:
Our immune system is daily challenged by a variety of fungal organisms. This requires a highly balanced immune defense, which provides protection without causing detrimental damage to the host, due to overreacting immune reactions. In mouse models, regulatory T cells (Treg) have been described to maintain tolerance at mucosal sites. However, the direct identification of antigen-specific human Treg against ubiquitous environmental antigens has so far been missing. Here we identified that specific Treg against the opportunistic fungal pathogens *Aspergillus fumigatus* and *Candida albicans* are strongly expanded in human peripheral blood. The data reveal further a unique capacity of the airborne fungus *A. fumigatus* to selectively trigger Treg responses, while maintaining a large part of the conventional T cell repertoire in a naive state. This Treg dominance is abrogated in cystic fibrosis patients with *Aspergillus* allergies, indicating an important contribution of the pathogen-specific Treg to Tcon ratio in the prevention of allergic immune reactions.

Author contribution:
Petra Bacher performed all experiments described in this manuscript, except of the TSDR analyses that were performed by the group of Birgit Sawitzki (Berlin-Brandenburg Center for Regenerative Therapies, Charité – University Medicine Berlin). Furthermore she was significantly involved in writing the manuscript.

Prof. Dr. Axel A. Brakhage

Manuscript IV

"The Human Th17 Cell Pool against Airborne Fungal Pathogens is Regulated by Cross-reactivity to Fungal Gut Microbiota."

Bacher P, Kniemeyer O, Hamprecht A, Assenmacher M, Cornely OA, Syrbe U, Maul J, Brakhage AA, Scheffold A.

Manuscript in preparation.

Summary:

The gut microbiota is known to have a major impact on local, but also on systemic immune homeostasis as well as immuno-pathology. One possible mechanism of how gut microbiota may influence gut-distal immune responses is *via* T cell cross-reactivity as a consequence of the extraordinary large amount of foreign microbial antigens. This manuscript shows, that the common human gut commensal *Candida albicans* triggers the expansion of a T cell population, which is cross-reactive to antigens of the airborne fungus *Aspergillus fumigatus*. This T cell cross-reactivity not only leads to a quantitative modulation of the *A. fumigatus*-specific T cell response, but importantly, also a qualitative modulation by the specific induction of Th17 cells in the otherwise Th1 biased *A. fumigatus* immune response. As Th17 responses in the lung are associated with a variety of lung disorders, these data might give an explanation of how the microbiota can influence the development of systemic diseases, through selective expansion of certain Th cell subsets with cross-reactivity for microbiota and gut-distal antigens.

Author contribution:

Petra Bacher performed all experiments described in this manuscript. Furthermore she was significantly involved in writing the manuscript.

Prof. Dr. Axel A. Brakhage

Manuscript V

"Rapid Diagnosis of Invasive Mold Infection by Measuring Fungus-Reactive $CD4^+$ T Cells in Peripheral Blood."

Bacher P*, Steinbach A*, Kniemeyer O, Hamprecht A, Assenmacher A, Vehreschild MJGT, Vehreschild JJ, Brakhage AA, Cornely OA*, Scheffold A*.

* These authors contributed equally to this work.

Manuscript in preparation.

Summary:

Invasive fungal infections (IFI) are life-threatening in immunocompromised patients leading to unacceptably high mortality rates. This is largely attributed to the lack of a timely and reliable diagnosis for IFI. This manuscript shows that the quantification of fungus-specific $CD4^+$ T cells by flow-cytometry in peripheral blood of immune-suppressed patients, allows a highly sensitive and specific discrimination between fungus infected and non-infected individuals. Fungus infected patients selectively show increased frequencies of fungus-specific T cells, compared to healthy controls or patients at risk, but without fungal infections. By analyzing the response against different fungal pathogens, discrimination between the invading fungal species was possible. Therefore, the detection of IFI by flow-cytometric measurement of fungus-specific T cell frequencies might enable the early and targeted anti-fungal treatment of infected patients and thus might strongly improve the non-satisfying situation of IFI diagnosis.

Author contribution:

In this equal contribution paper, Petra Bacher performed all experiments and was significantly involved in writing the manuscript. The clinical evaluation and classification of patients were performed by Angela Steinbach.

Prof. Dr. Axel A. Brakhage

Manuscript VI

"Identification of Immunogenic Antigens from *Aspergillus fumigatus* by Direct Multi-Parameter Characterization of Specific Conventional and Regulatory CD4$^+$ T Cells."

Bacher P, Kniemeyer O, Teutschbein J, Thoen M, Vödisch M, Wartenberg D, Scharf DH, Koester-Eiserfunke N, Schütte M, Dübel S, Assenmacher M, Brakhage AA, Scheffold A.

Manuscript submitted.

Summary:

Although a small population of *Aspergillus fumigatus*-specific CD4$^+$ T cells can be consistently detected in all healthy donors, it is currently unknown against which proteins these T cells react and which specificities are protective. Here, the antigen-specific CD4$^+$ T cell responses of healthy donors against various *A. fumigatus* morphotypes, subcellular fractions and selected single proteins were analyzed. The data indicate that a large part of the *A. fumigatus* immune response is directed against membrane proteins of the metabolically active developmental stages of the fungus. Further analyses of single *A. fumigatus* proteins allowed to define a set of immunogenic proteins, but display a broad and variable T cell response against various *A. fumigatus* proteins, independent of the subcellular localization, *i.e.* cell wall, membrane, cytosol or secretome. These data suggest that the complexity of the fungal proteome is reflected by the heterogeneity of the human T cell response and challenges the existence of one or few immune-dominant *A. fumigatus* proteins.

Author contribution:

Petra Bacher performed all experiments, except of the production of recombinant proteins, and was significantly involved in writing the manuscript. The recombinant CRF2 protein was generated by Mark Schütte (Department of Biotechnology, Technical University of Braunschweig). All *A. fumigatus* lysates were generated and all other recombinant proteins were identified and purified by collaborators from the Department of Molecular and Applied Microbiology, Hans-Knöll-Institute and Friedrich-Schiller-University Jena.

Prof. Dr. Axel A. Brakhage

3 Manuscripts

3.1 Manuscript I

"Flow-cytometric analysis of rare antigen-specific T cells"

Bacher P and Scheffold A.

Review article published in

Cytometry A. Aug; 83(8):692-701, 2013

REVIEW ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Flow-Cytometric Analysis of Rare Antigen-Specific T Cells

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• The cytometric enumeration and characterization of antigen-specific lymphocytes, as introduced about 15 years ago, has contributed significantly to our understanding of adaptive immune responses in health and disease. Despite the development of several technologies, allowing to directly or indirectly analyze many aspects of lymphocyte specificity and function, several unresolved issues remain, due to the low frequency of certain antigen-specific lymphocyte subsets and the complexity of T cell antigen recognition. This is especially true for $CD4^+$ conventional as well as regulatory T cells, which bring major contributions to immune protection and pathology. Here we review the current technologies for the analysis of antigen specific T cells within the physiologic T cell repertoire and with a special focus on recent technologies addressing the analysis of rare antigen-specific T cell populations including naive and regulatory T cells. © 2013 International Society for Advancement of Cytometry

• **Key terms**
antigen-specific T cells; rare cells; flow-cytometry; CD154; CD137; MHC multimer; tetramer; cytokine secretion assay; MACS

ANTIGEN-specific T cells play a central role in mediating specific immune responses as well as in the formation of immunological memory. Information about their frequencies, phenotypes, and functional capacities is essential to estimate the specific immune status of an individual, to understand the mechanisms of protective immunity or immunopathology and to predict immune protection or diagnose immune-related diseases. As a result of the high diversity of the T cell repertoire, allowing to respond to a myriad of different antigens, the frequency of T cells specific for a single peptide-MHC ligand is very low, in particular within the naive repertoire (range 0.2–60 cells/10^6 naive T cells)(1–19). But even within the memory repertoire, in the absence of acute infections, specific T cell frequencies in peripheral blood are typically well below 1%. Therefore, the analysis of antigen-specific T cells concerns the detection of rare events, demanding highly specific labeling methods which also allow processing of large cell samples to enable detection of sufficient numbers of the few antigen-specific T cells within the vast majority of nontarget cells.

Due to their low frequencies, antigen-specific T cells have been measured for decades only on the basis of antigen-specific proliferation, for example, via ^{3}H-thymidine incorporation. However, in such assays the phenotypic and functional properties of the reactive cells may be massively altered. Furthermore it is almost impossible to determine the actual frequency and phenotype of reactive cells in the starting cell sample and to exclude bystander proliferation. Therefore in this review, we focus on technologies allowing the direct quantification and characterization of antigen-specific T cells with only minimal in vitro manipulation. Flow cytometry has become the method of choice, since it combines the possibility to simultaneously measure a multitude of parameters for a single cell with the possibility to acquire high event numbers (~10^6). Therefore the development of several flow-cytometric methods for the identification of antigen-specific T cells has largely contributed to our current understanding of antigen-specific T cell responses by permitting a

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detailed phenotypical and functional characterization of each single detected cell. The possibility to measure 20 or more parameters from a single cell using polychromatic cytometry enables to gain maximal information from one measurement, which is essential for small sample sizes or rare subsets. For rare cell analysis the detection limit of flow-cytometric assays can further be decreased by combination with suitable pre-enrichment strategies, such as magnetic cell separation, which allow rapid processing of large cell numbers ($>>10^7$). In this way, a sufficient number of target cells can be collected for subsequent flow-cytometric characterization. Such technologies, allow the direct ex vivo detection and characterization of specific T cells for an unbiased knowledge about antigen-specific T cell responses. Here we review the current technologies for direct ex vivo flow-cytometric detection of antigen-specific T cells, including strategies to identify antigen-specific regulatory T cells and to access even extremely rare antigen-specific T cell populations, such as within the naive repertoire. We focus on technologies analyzing T cells within the physiologic repertoire, which are broadly applicable to human T cells. Technologies based on adoptive transfer of traceable numbers of antigen-specific T cells in mice are reviewed elsewhere (20).

Flow Cytometric Methods for the Ex Vivo Detection of Antigen–Specific T Cells

Detection methods can be classified into two categories, which together allow to access T cell specificity and function. Labeling of the specific TCR using recombinant MHC-peptide multimers identifies specific T cells directly according to their specific antigen receptor without restriction to certain functional parameters. This allows unbiased access to the total pool of T cells with a distinct specificity and within a certain affinity range (see below).

The second category employs certain functional parameters as a read-out for T cells, which react to specific antigen challenge. All relevant functions of T cells, such as cytokine release, expression of costimulatory molecules, cytotoxicity and proliferation are accessible via single-cell flow-cytometric assays and for most technologies also, selection markers on the cell surface are available allowing additional isolation of the specific cells.

Direct Labeling of Specific TCRs—MHC-Multimers

MHC-multimers have emerged as an important tool for the identification and characterization of antigen-specific T cells, by direct binding of the T cell receptor to its fluorescently labeled MHC-peptide ligand. Initial problems due to the low binding affinity of the T cell receptor to MHC-peptide monomers (21) have been overcome by the multimerization of peptide-MHC complexes to increase the relative binding avidity (22). Class I MHC-peptide multimers have widely been used to quantify and characterize antigen-specific $CD8^+$ T cell responses. The construction of class II multimers has initially been more difficult due to differences of the MHC class II structure and TCR affinity, but in the recent years a variety of MHC II multimers for the specific recognition of $CD4^+$ T cells have successfully been generated. In addition CD1 tetramers for detection of lipid reactive T cells including NKT cells have been developed (23–25).

The major limitation of the tetramer technology is that the antigenic epitope has to be characterized in detail, that is, a defined peptide restricted to a particular MHC haplotype. Peptide-exchange technologies allow rapid engineering of numerous different MHC-peptide reagents (26,27). Based on this, rapid epitope mapping techniques have been developed, which were successfully used to identify large numbers of new T cell epitopes (28–31). Furthermore, the use of combinatorial color coded tetramers enabled the simultaneous use of several tetramers to detect a greater number of different antigen-specificities (32,33). A distinct combination of fluorophores is used for each T cell specificity, allowing with n fluorophores a theoretical detection of $2^n - 1$ specificities. So far, this method has been shown to enable the detection of up to 63 different T cell specificities by the use of six different fluorophores (33).

Methods Analyzing T Cell Antigen-Reactivity

The MHC-multimer technology does per se not provide information on the functionality of the detected cells. But in particular, T helper cells can be subdivided in numerous subtypes solely defined by their specific set of effector functions, which are mostly acquired during first antigen encounter and form part of the immunological memory. Thus, information about the functional capacities of antigen-specific T cells and their classification into functionally different subsets is important to determine the quality of an immune response.

The detection of antigen-specific T cells by functional parameters requires the prior in vitro stimulation with the specific antigen. For stimulation, single peptides, proteins or whole antigen lysates can be used, as well as peptide pools, covering the whole sequence of a protein and all possible T cell epitopes. The possibility to use whole proteins and lysates is especially of interest to analyze T cell responses against complex antigens (e.g., whole pathogens or allergens), containing hundreds or thousands of different T cell epitopes. An advantage of activation dependent methods to enumerate antigen-specific T cells is that they are independent of MHC alleles or exact definition of the antigenic epitopes. However, the assays depend on the presence of a sufficient number of functional antigen presenting cells and per definition exclude anergic T cells from the analysis.

Cytokine detection allows identification of functional T cell subsets. The first reports about direct ex vivo enumeration of the frequencies of antigen-specific T cells by flow-cytometry utilized antigen-induced cytokine production (34–36). Cytokine expression is mostly confined to TCR activated T cells and it is transient, typically measured within 4–12 h, although within this time window different cytokines might have different kinetics. Cytokines can be detected either intracellularly when cells are simultaneously incubated with secretion inhibitors like Brefeldin A or Monensin (37,38) or on the cell surface by retention of the secreted cytokine on the surface

of the secreting cells via a capture matrix (34,39). The latter method has the advantage that surface retained cytokines enable the detection of live cytokine secreting cells as well as the enrichment via magnetic cell sorting. However, almost all cytokines are restricted to certain T cell subsets and in particular naive T cells produce only few cytokines upon stimulation. Therefore the enumeration of antigen-specific T cell frequencies solely based on cytokine production may be incomplete and has to be handled with care. On the other hand, the enrichment of highly specialized subsets can have significant advantages, for example, purified virus-specific IFN-γ secreting T cells are ideally suited for adoptive T cell therapy of viral infections (40–43).

Activation markers identify the total pool of reactive T cells. An alternative approach for the direct visualization of antigen-specific T cells by flow cytometry is the detection of activation markers, which are upregulated on the T cell surface upon antigen-specific triggering. Expression of some of these markers is independent of other effector functions like cytokine secretion or cytotoxicity, which may be restricted to the differentiation state of the T cells (e.g., naive, central memory, effector memory) or certain T cell subsets (e.g., Th1, Th2, Th17, etc.). Therefore activation markers permit the comprehensive characterization of the total pool of specific T cells against a given antigen, irrespective of functional specialization, MHC allele or exact definition of the antigenic epitope, as outlined above. A prerequisite for high specificity is that the marker expression should solely depend on T cell receptor triggering and expression should also be transient. A number of potential activation markers have been proposed, including CD69, CD25, CD71, HLA-DR, CD134 (OX40), CRTAM, CD137 (4-1BB), CD154 (CD40L) (44–55). However, a limitation for many of these markers is their sensitivity to bystander activation (CD69, CD25), their constitutive or exclusive expression on specialized T cell subset (CD69, CD25, CRTAM), or their late upregulation following in vitro stimulation (HLA-DR, CD134, CD71), which affects their usability for accurate enumeration of antigen-specific T cells occurring at low frequencies (<<1%).

One widely used activation marker for $CD4^+$ and $CD8^+$ T cells is CD69, which is one of the earliest markers (3–15 h; (44)) expressed on activated T cells, as well as B cells or NK cells (56–59). However, as CD69 background expression is also found in variable amount on nonstimulated T cells (49), and CD69 upregulation is not solely dependent on T cell receptor triggering (60,61), analysis of CD69 expression alone may strongly overestimate the frequencies of antigen-specific T cells. In contrast, in combination with other early activation markers (e.g., CD154 and CD137, see below) or cytokine expression, the analysis of CD69 coexpression as a second parameter is a useful tool to increase the sensitivity and optical discrimination of rare cells in flow-cytometry data.

For $CD4^+$ T cells, CD154 (CD40L), a member of the TNF superfamily, has been shown to be a reliable functional marker for the detection of antigen-activated T cells (45–47). As the central mediator of T cell help CD154 is expressed by virtually all functional activated $CD4^+$ T cells irrespective of their differentiation status, as well as by a subset of $CD8^+$ T cells. Another technically important feature of CD154 is its extremely low ex vivo background expression, which allows a highly specific detection of antigen-induced CD154 expression (62). This is most probably due to its rapid internalization and degradation following interaction with its receptor CD40 expressed on antigen-presenting cells. CD154 expression can readily be assessed either intracellularly by adding secretion inhibitors like Brefeldin A or Monensin to the culture or on the surface by blocking the interaction with its ligand through addition of an anti-CD40 mAB. As CD154 upregulation occurs fast (4–12 h) following antigen encounter, its detection can easily be combined with staining for cytokines and phenotypic markers.

Another well described marker with sufficiently high specificity is CD137 (4-1BB), a member of the TNFR superfamily, which has been shown to be expressed on antigen-activated $CD4^+$ and $CD8^+$ T cells as well as $\gamma\delta^+$ T cells, following 16–24 h of stimulation (51–53). Recently, CD137 has also been shown to be expressed on antigen-activated $CD4^+Foxp^+$ regulatory T cells (Treg, see below) (63). Since both, CD154 and CD137 are expressed on the surface of the antigen-specific T cells, enrichment and analysis of living cells is possible allowing further functional characterization or clinical-scale enrichment of antigen-specific T cells for adoptive therapies.

Accessing Rare Antigen–Specific T Cells

Despite its high sensitivity, standard flow-cytometry is limited by the number of events, which can be acquired (typically $\sim 10^6$ cells) as well as the staining background, which is typically between 0.01 and 0.1%. This usually restricts the analysis of un-manipulated samples to populations occurring at frequencies >0.01–0.1%. Therefore, the technologies described above have mainly be used for the detection of pathogen specific memory T cells, although the frequency of pathogen-specific T cells can vary widely, depending on the nature of the pathogen, the status of the immune response and the persistence or clearance of the pathogen. In absence of an acute infection, frequencies of antigen-specific memory cells are typically in the range of one cell within 100 to 10^5. However, many functionally important T cell populations occur at much lower frequencies and cannot be accessed without additional measures. This applies in general for the naive compartment, as well as the $CD4^+$ T cell repertoire and in particular for the small population of regulatory T cells. Furthermore T cells specific for nonpathogen derived antigens, that is, auto-antigens, tumor antigens, environmental antigens (allergens, toxins, food) or neo-antigens are typically below 0.01% (Fig. 1). Methods to enumerate such rare populations must therefore allow to process large cell numbers, to collect sufficient target events for statistical relevant analyses and at the same time prevent accumulation of background events.

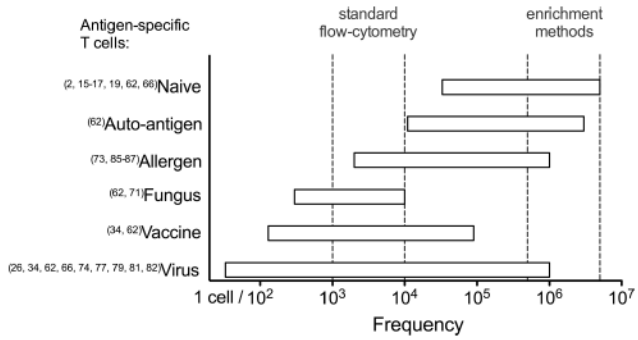

Figure 1. Frequencies of antigen-specific $CD4^+$ and $CD8^+$ T cells within human peripheral T cell repertoire determined directly by ex vivo enrichment methods. Dashed lines indicated the detection limit range of standard flow cytometric counting and magnetic enrichment methods, respectively.

In Vitro Expansion of Antigen-Specific T Cells to Facilitate Detection

One solution to the rare cell problem is to increase the number of antigen-specific T cells by in vitro expansion methods. Numerous approaches have been published using cultivation of un-separated PBMC with the particular antigen for 1–2 weeks allowing the specific T cells to grow preferentially. However, the expansion of a single T cell is affected by a number of hardly predictable parameters such as its functional status (e.g., naive, memory, anergic), the presence of other reactive cells (bystander activation), or the relation between the number of cell divisions and cell death. Therefore, it is difficult to extrapolate from the frequencies obtained after prolonged in vitro culture to the frequencies of cells in the original samples, even if the proliferative history of the cells has been visualized, for example, by use of cell proliferation dyes. Similarly, the phenotype and function of the expanded cells may significantly be altered by the culture conditions.

The group of Sallusto recently described the use of libraries of polyclonal expanded $CD4^+$ T cells, to determine the frequencies of antigen-specific T cells within the human naive and memory repertoire (64). Each library consists of several hundred single cultures, each seeded with 2,000 T cells cells/well, so that at the most one specific T cell/well could be expected. The cells are polyclonally expanded while maintaining the TCR diversity and restimulated with one or several antigens in the presence of autologous monocytes. The original frequency of antigen-specific T cells can then be calculated based on the number of proliferating cultures and the number of input cells. Using this approach, the authors could detect frequencies of antigen-specific naive $CD4^+$ T cells for protective antigen from *Bacillus anthracis* between 10 and 26 per 10^6 naive $CD4^+$ T cells and for KLH between 10 and 70 cells per 10^6 naive $CD4^+$ T cells. An advantage of this method is that it enables the enumeration of antigen-specific T cells specific for naturally processed antigens. By presorting of different T cell subpopulations for set-up of the libraries, antigen specific T cell frequencies from various T cell compartments can be determined and compared. Once generated, the libraries can be used for screening of several antigens and for further functional analyses such as TCR affinity determination. However, the technique requires maintaining several hundreds of individual cultures for several weeks. Furthermore, a bias through selective outgrowth or loss of certain T cell clones cannot be excluded, especially for mixed starting populations, for example, different memory subsets, due to differential expansion and survival potential. Finally, the detection limit is determined by the number of individual cell cultures per library and the number of input cells per culture. For a frequency of one cell within 10^5 only 10 positive wells are detected within 500 cultures (i.e., 2,000 cells/culture, 10^6 cells in total). This basically restricts the analysis to a frequency window from 1 cell within 2,000 (all cultures positive) to maximally one cell within 10^5 (10 cultures positive). For example the authors fail to detect the rare specific T cells against neo-antigens within the memory T cell compartment, which have been previously described (65) and can be readily detected by other approaches (62,66).

Antigen-Specific Pre-Enrichment

Magnetic enrichment to collect few target cells from large cell samples. An alternative approach to increase the number of rare antigen-specific T cell frequencies for proper cytometric analysis utilizes quantitative pre-enrichment of target cells via magnetic cell separation, which allows rapid processing of large cell samples (10^6–10^9) (67–70). In this way, all labeled antigen-specific T cells (e.g., peptide-MHC tetramer, activation marker, surface captured cytokines) from a large sample are retained on the column and can subsequently be conveniently analyzed by flow-cytometry or used for further functional studies or expansion. A prerequisite for the magnetic enrichment of antigen-specific T cells is a high specificity of the sorting marker to reliable identify the few antigen-specific T cells within the vast number of irrelevant cells. As the eluted fraction still contains significant frequencies of nontarget cells, additional exclusion criteria, such as staining of non-T cell lineage cells, doublets and dead cells are required to increase sensitivity and specificity. In combination with multi-parameter flow cytometry, this technology allows the direct ex vivo detection and in depth characterization of few antigen specific T cells from large sample sizes. The magnetic enrichment of antigen-specific cells leads to a dramatic increase of sensitivity, which is mainly limited by the number of available input cells, and gives important new insights into previously undetectable parts of the T cell repertoire, including even the naive compartment. The general characteristics of different enrichment methods for the detection of antigen-specific T cells are summarized in Table 1.

Enrichment of cytokine secreting cells. The enrichment of rare cytokine producing T cells, enabled by the cytokine secretion assay was the first report utilizing magnetic enrichment for the analysis of rare antigen-specific T cells undetectable via conventional flow-cytometry (34).

This has been extended to several other rare cytokine producing T cell subpopulations. For example, tetanus-

Table 1. Enrichment methods for the detection of rare antigen-specific T cells

METHOD	ADVANTAGES	DISADVANTAGES	DETECTABLE T CELL POPULATIONS
Direct detection			
MHC-multimer	• Activation independent • High specificity	• Knowledge/availability of MHC and epitope required • Restricted to single epitope specificities • Does not reveal functional status of the cells • Detection of low-affinity cells difficult	Naive, memory, Treg
Activation dependent	• No MHC/epitope restriction • Reveal functional status	• Dependent on T cell activation	
Cytokine secretion assay	• Selective isolation of distinct cytokine producing subsets	• Restricted to few selected cytokine producers • Laborious • Risk of contaminating cells through cross-feeding	Memory
CD154 (6 h)	• Detection of the whole antigen-specific CD4+ T cell response • Fast kinetics • Compatible with cytokine analysis	• Mainly restricted to CD4+ T cells	Naive, memory
CD137 (16 h)	• Detection of the whole antigen-specific CD4+, CD8+ T cell response	• Not compatible with cytokine analysis • No differentiation between Treg and conventional CD4+ T cells (due to 16 h stimulation)	Naive, memory
CD154/CD137 (6 h)	• Detection of the whole antigen-specific CD4+ conventional and regulatory T cell response	• No CD8+ T cells	Naive, memory, Treg

specific IFN-γ^+ and IL-4$^+$ CD4$^+$ T cells or *C. albicans*-specific IL-17$^+$ CD4$^+$ T cells were isolated with frequencies of approximately one cell within 10^4–10^5 PBMC (34,71) and autoreactive IFN-γ^+ and IL-4$^+$ CD4$^+$ T cells in pemphigus vulgaris patients could be detected with frequencies of 3–40 cells per 10^5 PBMC and less than 10 cells per 10^5 PBMC in healthy individuals (72). In addition, rare allergen-specific IL-4, IFN-γ, and IL-10 producers have been isolated from allergic patients and healthy individuals with frequencies of 1–10 cells within 10^4 CD4$^+$ T cells (73). The direct visualization of allergen-specific cytokine secreting cells revealed a higher frequency of IL-10 producing cells in healthy individuals, whereas in allergic patients IL-4 producers were dominant indicating that the balance between IL-10 and IL-4 producing cells might be decisive in the development of allergy (73).

Enrichment of rare IFN-γ producing cells has also been instrumental for clinical adoptive immunotherapy, for example, clinical-scale isolation of rare virus-specific T cells (40–43).

However, as outlined above cytokine secretion is heterogeneous and restricted to certain T cell subsets, which often represent only a small fraction of the total antigen-specific T cells. Although the cytokine secretion assay allows simultaneous detection of more than one cytokine, it is limited to maximally two or three, since the labeling of an individual cell with different catch matrices for the respective cytokines reduces the labeling intensity, which precludes their proper identification as well as magnetic enrichment.

Tetramer enrichment. MHC tetramers were also used to detect and isolate rare antigen-experienced CD4$^+$ and CD8$^+$ memory T cells of humans and mice (26,74–82). The sensitivity of the tetramer-based enrichment technology was further extended to the identification and first direct enumeration of the extremely rare antigen-specific CD4$^+$ and CD8$^+$ T cells within the naive T cell repertoire. In the murine system, naive CD8$^+$ T cells specific for epitopes of ovalbumin or various viruses could be detected with varying frequencies from 15 to 1,500 cells per mouse (7–14,18). The peptide-MHC class II tetramer based enrichment revealed frequencies of naive CD4$^+$ T cells specific for epitopes within ovalbumin, *Salmonella typhimurium*, *Listeria monocytogenes*, I-E alpha chain or the interphotoreceptor retinoid binding protein (IRBP) from 20–260 cells per mouse (1–6). Given an average number of 2.5 $\times$ 10^7 naive CD8$^+$ and 3.5 $\times$ 10^7 naive CD4$^+$ T cells per mouse (83), the identified frequencies of T cells for an individual peptide-MHC ligand range from 0.6 to 60 cells per 10^6 naive CD8$^+$ T cells and 0.6–7.4 cells per 10^6 naive CD4$^+$ T cells. Also in humans, antigen-specific naive T cells were enumerated by tetramer-based enrichment with surprisingly

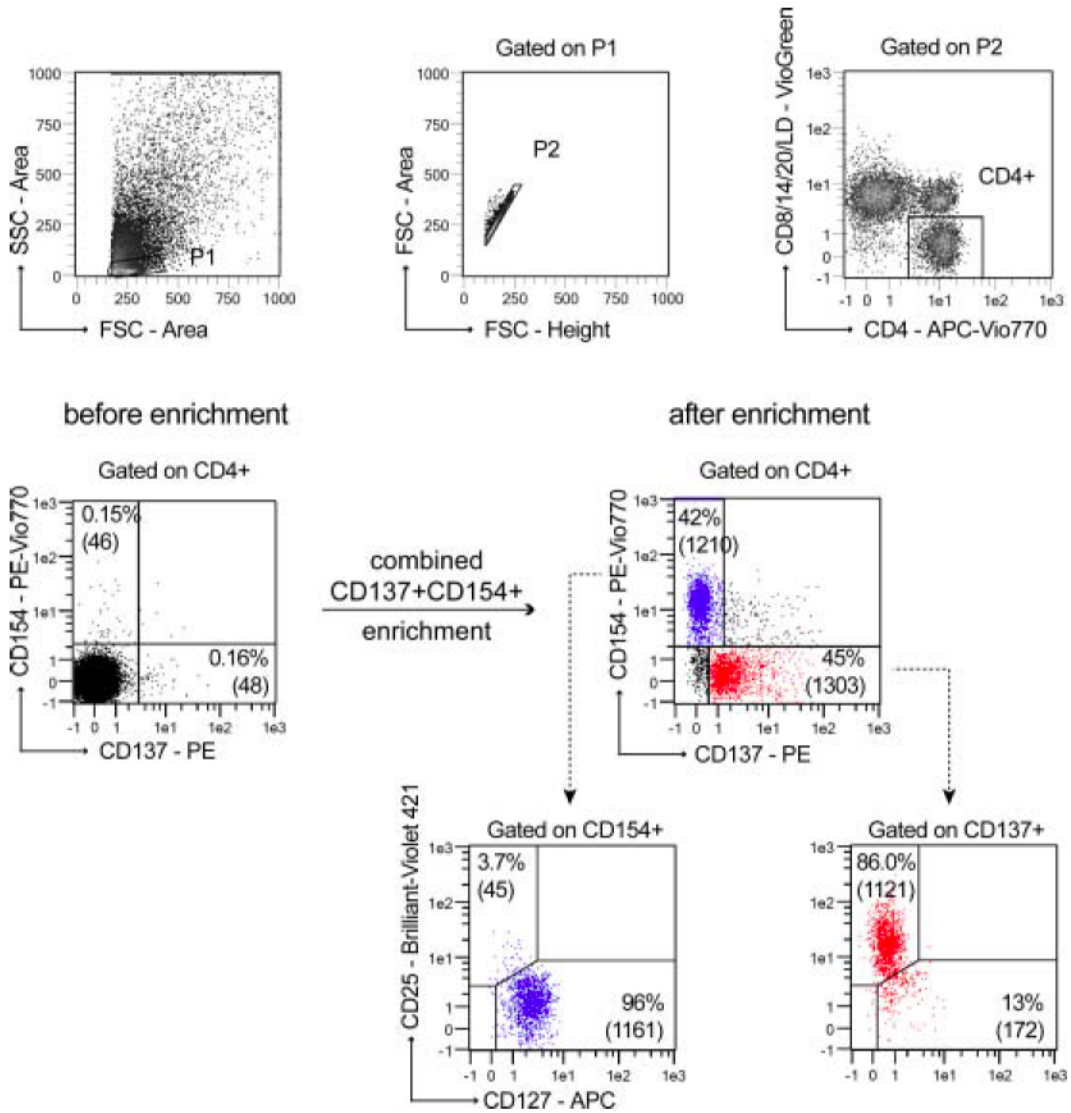

Figure 2. Detection of antigen-specific conventional $CD4^+$ T cells and regulatory T cells following combined magnetic enrichment of $CD154^+$ and $CD137^+$ cells. PBMC of a healthy human volunteer were stimulated for 6 h with *A. fumigatus* lysate. $CD154^+$ and $CD137^+$ $CD4^+$ T cells were isolated by magnetic enrichment and counterstained for CD25 and CD127 expression. For an optimal detection of CD4+ CD154+ and CD137+ events, cell aggregates (scatter area versus scatter height), dead cells and nontarget cells ($CD8^+$ $CD14^+$, $CD20^+$, dump) were excluded and cells were gated on $CD4^+$ lymphocytes. Numbers in brackets indicated cell count within 2.5×10^5 analyzed PBMC before enrichment and following enrichment from 1×10^7 PBMC. FSC, forward scatter; SSC side scatter; LD, live/dead; PE, phycoerythrin; APC, allophycocyanin. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

similar frequencies: $CD4^+$ T cells specific for a peptide of influenza hemagglutinin and three different epitopes of *Bacillus anthracis* ranged between 0.2 and 10 cells per 10^6 naive $CD4^+$ T cells (2,16), which is in the same range as estimated by the library method (64). Within the $CD8^+$ T cell repertoire antigen-specific naive T cells for epitopes of different viruses and the auto-antigen NY-ESO1 were analyzed, with frequencies of 0.5–30 per 10^6 naive $CD8^+$ T cells (15,17,19).

An interesting finding of these studies was that the frequencies of naive precursor T cells for different antigens can vary widely, but showed limited inter-individual variation. This is especially surprising in humans, as HLA haplotypes are highly diverse. Furthermore, the size of the naive T cell population specific for a given antigen could be linked to immune dominance (13,84), as it was shown that naive precursor frequencies correlate with the size of the memory T cell response (4,10,13,16,19,84).

Only recently a completely different mechanism for shaping the human memory repertoire has been highlighted by Davis and coworkers (66). They used highly sensitive HLA class II tetramer enrichment to show that the peripheral blood of human individuals which have not been infected with a certain virus before contain significant frequencies of bona fide memory T cells specific for the very same virus, suggesting that cross-reactivity during life contributes significantly to shaping of the immune repertoire. Also our own data, using

ex vivo enrichment of CD154 expressing cells following auto-antigen stimulation suggest, that a significant proportion of the self-reactive $CD4^+$ T cell pool in healthy individuals has a memory phenotype, which may result from cross-reactivity to other antigens ((62) and see below).

Tetramer based enrichment has also been used to analyze rare allergen-specific T cells as potential correlates for disease status or efficacy of immunotherapy. Because of their low frequencies, previous analyses of allergen specific T cells using MHC-multimers were mainly performed after in vitro expansion, which can alter the phenotype and functional capacities of the cells, as already discussed above. Direct ex vivo tetramer enrichment of allergen-specific $CD4^+$ T cells has recently been used to analyze specific T cells against epitopes from cat, peanut, alder, and birch allergens (85–87). Allergen-specific T cells were barely detected in peripheral blood of nonatopic individuals with frequencies of about one to five cells within 10^6 $CD4^+$ T cells. In contrast, average–frequencies in allergic patients ranged from 7–500 cells within 10^6 $CD4^+$ T cells. The differences in the frequencies of allergen-tetramer positive cells might reflect, that allergen specific T cells are present at lower frequencies in nonallergic subjects. Alternatively, a lower avidity of allergen-reactive T cells in healthy individuals has been suggested (88), and tetramers may be limited in their ability to detect low-affinity T cells (89). Although target proteins and T cell epitopes are defined for many allergic diseases, the tetramer technology restricts the analysis to a few preselected epitopes and MHC haplotypes and might therefore underestimate the frequencies of allergen specific T cells in healthy donors. In fact, our own preliminary results, analyzing the total allergen-reactive T cell pool using CD154 enrichment after stimulation with whole proteins or allergen extracts (see below), indicate no difference in specific T cell numbers between healthy and allergic subjects but a modulation of memory T cell subsets, as well as a strong shift towards a Th2 phenotype (Bacher et al., unpublished).

Rare antigen-specific $CD4^+$ T cell analysis using CD154 enrichment. The advantages of CD154 as a marker for the total pool of antigen-reactive T cells were discussed above. We recently demonstrated that CD154 enrichment can also be utilized to strongly increase the sensitivity of detection for rare cells (10^{-5}–10^{-6} largely depending on the number of input cells) (62). Using this approach, a broad functional heterogeneity of virus-, bacteria- or fungus-specific $CD4^+$ T cells was visualized, demonstrating that many functional relevant cytokines occur only in very low frequencies within the total antigen specific T cell pool, undetectable without pre-enrichment methods (1–10% of $CD154^+$ cells, which corresponds to 0.0001–0.1% within the $CD4^+$ T cell repertoire). Despite this heterogeneity, these "cytokine micro-patterns" were characteristic for a specific pathogen, indicating a pathogen-specific immune modulation, which cannot be classified according to the simplified Th1, Th2, Th17 lineage differentiation scheme. The increased sensitivity through the magnetic enrichment was further used to directly analyze the full repertoire of KLH-, HIV- or several auto-antigen-specific $CD4^+$ T cells in the peripheral blood of healthy donors. The frequencies of these cells were in the range of 1 cell within 10^4 to 10^6, which is similar to the results obtained using the "T cell library approach" (64). However, we could furthermore demonstrate that about on third of the identified neo- or auto-antigen-specific $CD154^+$ T cells in healthy donors had a memory phenotype, suggesting that these cells are the result of priming by cross-reactivity, which extends the findings by Mark Davis group for virus-specific T cells using tetramer enrichment (66).

Antigen–Specific Regulatory T Cells

A further challenge is the detection and enumeration of antigen-specific $CD4^+Foxp3^+$ regulatory T cells (Treg). Despite their importance for the regulation of immune responses and the maintenance of tolerance, exact information of antigen-specificity and the frequency of antigen-specific Treg is lacking. Fragmentary knowledge of specific target antigens or even antigenic peptides limits the applicability of MHC class II multimers. In addition, less is known about the affinity of Treg cells to their peptide-MHC ligands, which might differ from conventional T cells and may be below the detection threshold of MHC class II multimers. Treg also lack most of the effector functions typically used for the analysis of conventional T cells, such as cytokine production or in vitro proliferation. Finally, sensitive analysis tools are particularly relevant for antigen-specific Treg, since they usually represent a subset of only 5–10% within the circulating human $CD4^+$ T cell compartment but have similar TCR diversity, which means that antigen-specific Treg are extremely rare. Therefore, enrichment strategies are essential for their reliable detection in nonmanipulated biological samples.

Tetramer Detection of Treg Cells

MHC class II tetramers have been successfully used to analyze antigen-specific Treg cells in studies of autoimmunity, allergy, transplantation or pathogen-specific immune responses. A recent study in mice addressed the question of thymic selection impact on $Foxp3^+$ and $Foxp3^-$ self-peptide specific $CD4^+$ T cells, using tetramer enrichment (90). Self-peptide specific $CD4^+$ T cells were not deleted in mice that ubiquitously expressed the antigen, but both $Foxp3^-$ and $Foxp3^+$ subsets were reduced compared to wildtype mice. However, the remaining T cells were enriched for $Foxp3^+$ cells, indicating a higher resistance of $Foxp3^+$ T cells to negative selection. Another important finding of this study was that Treg cells specific for foreign antigens could be detected in the $CD4^+$ T cell repertoire of mice, that were never exposed to the antigen before (90,91). The frequencies of $Foxp3^+$ cells specific for epitopes of I-E α-chain, *Lymphocytic choriomeningitis virus, Listeria monocytogenes* or ovalbumin ranged from 7.5 to 12% of the respective specificities in the naive T cell repertoire (90).

In humans MHC class II tetramers were used to identify antigen-specific Treg cells for birch, alder, and timothy grass

allergens, as well as epitopes from *Streptococcus pneumonia, Varicella zoster, Mycobacterium tuberculosis,* Hepatitis C Virus, Influenza, HIV or the islet specific antigens GAD and IGRP (87,92–99). However, due to their low frequencies, in most of these studies antigen-specific Treg cells were previously expanded for several days, by stimulation of bulk cultures with the respective antigenic epitopes. Under these conditions, differentiation between Treg and conventional T cells is difficult, since Treg specific markers such as CD25 and Foxp3 or lack of CD127 expression are also acquired by non Treg cells upon activation (100). This further underlines the importance to analyze and enumerate antigen-specific Treg cells directly ex vivo.

Recent studies using MHC II tetramers have demonstrated directly ex vivo the existence of human regulatory T cells specific for foreign antigens. Foxp3$^+$ Treg cells specific for a peptide of the major birch pollen allergen Bet v1 were detected in human tonsils with frequencies of approximately one cell within 1,000 CD4$^+$ T cells, which corresponded to about 30% of the total tetramer positive population (93). However, in peripheral blood no specific Treg cells could be detected. Similarly, tetramer positive *Streptococcus pneumonia* specific Foxp3$^+$ Tregs could be detected in human tonsils, with frequencies ranging from 0.53 to 1.96% among the total tonsillar Treg cell population, whereas among peripheral blood Treg cells no tetramer positive cells were found (96). The different Treg frequencies in tonsils *versus* peripheral blood argue for a selective accumulation of foreign antigen-specific Treg cells at the site of antigen encounter. Also after cutaneous *Varicella zoster* or *Mycobacterium tuberculosis* challenge, human antigen-specific Tregs were identified by specific tetramers at the site of antigen-challenge in the skin (97). However, when tetramer enrichment was used, antigen-specific Tregs against Hepatitis C virus, Influenza and alder allergen could directly be detected in human peripheral blood with frequencies of ~5–15% of the total tetramer positive population (87,95), which is surprisingly similar to the 7.5–12% Treg cells within the foreign peptide-specific naive T cell repertoire detected in mouse (90). These data in fact suggest that Treg are part of the natural repertoire against foreign antigens and that specific Treg coexpand together with conventional T cells during an immune response most likely to control or prevent excessive immune reactions.

Treg Specific Activation Markers

Although the typical markers of TCR-activated conventional T cells are lacking on Treg cells, several Treg specific activation-markers have been introduced that allow the isolation of activated Treg cells after in vitro stimulation, including CD121a/b, latency-associated peptide (LAP) and GARP (LRRC32) (101–103). But so far none of these markers has been evaluated for an antigen-specific analysis of Treg cells. However, only recently the activation markers CD154 and CD137 have been reported to be upregulated on Treg cells following short term in vitro antigen stimulation (63,104). Schönbrunn et al. show that the kinetics of activation marker expression differ between Treg and conventional T cells, that is after 6 h stimulation only Tregs express CD137 whereas conventional T cells require >12 h of stimulation (63). In this study, also the relation between CD154 and CD137 expression was analyzed. CD137 is expressed by all activated Tregs, whereas CD154 expression is restricted to a subset of Treg cells which actually seem to represent a population with instable Foxp3 expression as shown by methylation analysis of the Foxp3 promotor region. Thus the combined analysis of CD137 and CD154 following short-term (6 h) stimulation might be optimal to detect in parallel conventional T cells and Tregs reacting against the same antigen and allows even to discriminate between subsets with stable (CD137$^+$CD154$^-$) or unstable (CD137$^+$ CD154$^+$) Foxp3 expression. Schönbrunn et al. used the technology to directly sort highly suppressive alloreactive Tregs out of PBMC stimulated with allogenic APCs.

Since both markers can be accessed on the cell surface they can also be used in combination with a magnetic preenrichment of the activation marker positive cells (62). This approach thus enables the detection of rare antigen-specific cells with high sensitivity, but without limitations of TCR affinities or restriction to certain epitopes and could therefore be extended to any antigen of interest. Our own preliminary results using a combined CD137/CD154 enrichment indeed show that antigen-specific Treg can be detected with high sensitivity (Fig. 2) and we are currently using this approach to characterize antigen-specific Treg cells from human peripheral blood directed against environmental antigens, as well as auto-antigens.

Conclusion

Recent developments which significantly increase the sensitivity for cytometric detection of antigen-specific T cells and also enable the detection of antigen-specific Tregs, now allowing to analyze the full CD8$^+$ as well as CD4$^+$ T cell repertoire against most pathogens, environmental antigens, and tumor- or auto-antigens, even in the unexposed host. In particular, the possibility to simultaneously analyze T cell responses in the naive, effector as well as regulatory T cell compartment will help to define their role within the immune network and to deduce their lineage relationship. Understanding of the full antigen-specific repertoire will significantly improve diagnostic and prognostic as well as therapeutic intervention strategies.

Although the various technologies yield comparable results in terms of cell frequencies they highlight different aspects of T cell specificity and function and therefore integrative approaches are mandatory. The main limitation seems to be the availability of sufficient sample material, especially from patients, which allows to collect a suitable number of target cells. The small number of target cells also makes high demands on downstream analysis tools and therefore multiparameter analysis tools such as polychromatic flow-cytometry will be essential to extract maximal information from the few available cells.

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3.2 Manuscript II

"Antigen-Reactive T cell Enrichment for Direct, High-resolution Analysis of the Human Naive and Memory Th Cell Repertoire"

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Antigen-Reactive T Cell Enrichment for Direct, High-Resolution Analysis of the Human Naive and Memory Th Cell Repertoire

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Ag-specific CD4$^+$ T cells orchestrating adaptive immune responses are crucial for the development of protective immunity, but also mediate immunopathologies. To date, technical limitations often prevented their direct analysis. In this study, we report a sensitive flow cytometric assay based on magnetic pre-enrichment of CD154$^+$ T cells to visualize rare Ag-reactive naive and memory Th cells directly from human peripheral blood. The detection limit of ~1 cell within 10^5–10^6 permitted the direct enumeration and characterization of auto-, tumor-, or neo-Ag–reactive T cells within the naive and even memory CD4$^+$ T cell repertoire of healthy donors. Furthermore, the analysis of high target cell numbers after pre-enrichment of rare Ag-specific T cells from large blood samples dramatically improved the identification of small subpopulations. As exemplified in this work, the dissection of the Ag-specific memory responses into small cytokine-producing subsets revealed great heterogeneity between pathogens, but also pathogen-related microsignatures refining Th cell subset classification. The possibility to directly analyze CD4$^+$ T cells reactive against basically any Ag of interest at high resolution within the naive and memory repertoire will open up new avenues to investigate CD4$^+$ T cell–mediated immune reactions and their use for clinical diagnostics. ***The Journal of Immunology*****, 2013, 190: 3967–3976.**

T helper cells play a central role in the induction of immune responses against pathogens as well as in the development of immune-mediated diseases such as autoimmunity or allergy. T cells recognizing protective or disease-relevant Ags are usually rare, especially in the naive repertoire, but information about their frequency, phenotype, and functional capabilities has important diagnostic and/or prognostic value. Frequencies of Ag-specific T cells within the human naive repertoire have been estimated to range between 1 cell in 10^4–10^7 (1–3). In the memory repertoire, the frequencies strongly vary depending on the status of the immune reaction and are usually in the range between 10^{-5} and 5%. Recent efforts to define correlates of immune protection, for example, after vaccination, have revealed that multiparameter classifications are crucial, for example, for the identification of multifunctional CD8$^+$ T cells (4–6). This imposes the need to analyze sufficient target events to be able to identify small subsets with high statistical significance.

Several flow cytometric methods for the direct detection of rare Ag-specific T cells have been described in the recent years, including peptide-MHC multimers, cytokine secretion, and expression of activation markers (7, 8). However, these technologies are limited by the number of cells per sample that can be acquired in reasonable time (10^5–10^6) as well as by the natural and/or technological background of the assay, which is typically between 0.01 and 0.1%. T cells occurring at lower frequency have traditionally been analyzed via indirect methods, such as long-lasting in vitro cultivation and/or population-based methods ([^{3}H]thymidine incorporation, CFSE dilution, ELISA) or ELISPOT (9–14). These techniques provide only limited information about the relationship between frequency, phenotype, and function of the reactive cells.

Recent studies have shown that magnetic enrichment can be used to collect rare peptide MHC-multimer–labeled cells from large sample sizes, allowing direct cytometric detection of murine and human Ag-specific CD4$^+$ and CD8$^+$ T cells even in the naive repertoire (1, 15–22). However, the MHC-multimer technology is limited to the previous knowledge of MHC alleles and antigenic epitopes. Due to the very high diversity of MHC molecules and especially for complex pathogens, containing hundreds or thousands of different target epitopes, choosing the right peptide-MHC combinations to study Ag-specific T cell responses is quite challenging (23). Furthermore, the restriction to few antigenic epitopes gives only limited and preselected insight into the heterogeneous T cell populations specific for a certain Ag. In addition, the generation of functional peptide-MHC class II multimers for the detection of Ag-specific CD4$^+$ T cells, especially for low-affinity autoreactive cells, is still difficult.

A more recent approach used libraries of polyclonal expanded naive or memory CD4$^+$ T cells to determine the frequencies of Ag-specific T cells against naturally processed Ags (2). This method

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The online version of this article contains supplemental material.

Abbreviations used in this article: AdV, adenovirus; ARTE, Ag-reactive T cell enrichment; GAD, glutamic acid decarboxylase; KLH, keyhole limpet hemocyanin; MOG, myelin oligodendrocyte glycoprotein; WT-1, Wilms tumor Ag.

does not require knowledge of peptide-MHC combinations and allows detection of the total pool of Ag-specific T cells even for complex organisms in the memory as well as the naive $CD4^+$ T cell compartment. However, a disadvantage of this technique is that it requires several weeks of in vitro cultivation and does not allow directly enumeration and characterization of the Ag-specific T cells, which implies the risk of potential phenotypic or functional changes introduced by in vitro manipulation.

To overcome the current limitations, we developed a method based on magnetic enrichment of Ag-reactive $CD154^+$ (CD40L)–expressing T cells to access directly ex vivo the human Ag-specific naive and memory $CD4^+$ T cell repertoire. CD154, which is specifically expressed by all Ag-activated $CD4^+$ T cells shortly after TCR stimulation, is a reliable functional marker for Ag-specific $CD4^+$ T cells irrespective of MHC allele or exact definition of the antigenic epitope (24–26). We developed an optimized magnetic Ag-reactive T cell enrichment protocol (ARTE) of $CD154^+$ T cells, which allows reproducible and highly sensitive capture of rare Ag-specific $CD4^+$ T cells prior to their phenotypical and functional in-depth characterization by multiparameter flow cytometry. The high sensitivity of our method provides direct access to the human naive T cell repertoire and, to our knowledge, enabled the first characterization of human $CD4^+$ T cells specific for an unrestricted pool of T cell epitopes from various auto- and tumor-associated Ags, directly ex vivo from peripheral blood of healthy donors. By performing functional in-depth characterizations, we dissected the Ag-reactive $CD4^+$ T cell memory responses against various pathogens into small cytokine subsets and identified a high functional heterogeneity with additional pathogen-related patterns not necessarily associated with the dominant Th1, Th2, or Th17 classification.

Our technology will be broadly applicable and strongly improve the analysis of autoimmunity and allergies, as well as tumor immunology and infection research, and will greatly refine $CD4^+$ T cell monitoring during vaccinations and other immunotherapies.

Materials and Methods

Blood donors and isolation of PBMCs

Buffy coats or leukaphereses products from healthy donors were obtained from the university hospital in Dortmund and Cologne. This study was performed according to established ethical guidelines, and all blood donors gave informed consent. PBMCs were separated by use of Ficoll–Hypaque (GE-Healthcare, Bio-Science) density gradient centrifugation.

Preparation of Aspergillus fumigatus *lysate*

The *A. fumigatus* strain ATCC 46645 (LGC Standards) was used for preparation of protein extract from mycelium. Conidia were inoculated at a concentration of 2×10^6 spores/ml in YPD medium and shaken for 20 h at 37°C with 200 rpm (27). Mycelium was recovered by filtration, washed with water, and stored at −70°C. Frozen mycelium was resuspended in saline (0.9% [w/v] NaCl) and disrupted in a microdismembrator (Sartorius) at 200 rpm for 10 min using glass beads. The extract was resuspended in 1× PBS buffer (2 mM $MgCl_2$) and centrifuged for 20 min at 20,000 × *g*. Supernatant was stored in aliquots at −20°C until use.

Stimulation, isolation, and characterization of Ag-specific T cells

Stimulations were performed in RPMI 1640 medium (Miltenyi Biotec), supplemented with 5% (v/v) AB serum (Lonza) and 2 mM L-glutamine (PAA Laboratories). For enrichment of recall Ag-specific T cells, 1×10^7 PBMCs were stimulated for 7 h with the following Ags: *A. fumigatus*-lysate (40 μg/ml), *Candida albicans*-lysate (20 μg/ml; Greer Laboratories), CMV-lysate (10 μg/ml; Siemens), tetanus-toxoid (10 μg/ml; Statens Serum Institute), or peptide pools of CMV pp65, adenovirus (AdV) Hexon (0.6 nmol/ml; both Miltenyi Biotec) in the presence of 1 μg/ml CD40 and 1 μg/ml CD28 functional grade pure Ab (both Miltenyi Biotec). For enrichment of auto-/tumor-/neo-Ag–specific T cells, 1×10^8 PBMCs were stimulated with keyhole limpet hemocyanin (KLH; 200 μg/ml; Immuchothel Biosyn) or peptide pools of *C. albicans* MP65, myelin oligodendrocyte glycoprotein (MOG), glutamic acid decarboxylase (GAD), NY-ESO, Wilms tumor Ag (WT-1; 0.6 nmol/ml; all Miltenyi Biotec), and HIV Gag (1 μg/ml; JPT Peptide Technologies). If separation of Ag-specific T cells was combined with intracellular staining, 1 μg/ml brefeldin A (Sigma-Aldrich) was added for the last 2 h of stimulation. After stimulation, cells were separated using the CD154 MicroBead Kit (Miltenyi Biotec). In brief, cells were indirectly magnetically labeled with CD154-biotin and antibiotin Microbeads and enriched by two sequential MS MACS columns (Miltenyi Biotec). If separation of Ag-specific T cells was combined with an intracellular staining, surface staining was performed on the first column, followed by fixation, permeabilization (Inside stain kit; Miltenyi Biotec), and intracellular cytokine staining on the second column.

HLA-DR–blocking experiments and fixation of APCs

A total of 1×10^7 PBMCs from CMV-seropositive donors was stimulated in presence or absence of a HLA-DR–specific mAb (100 μg/ml clone AC122; Miltenyi Biotec). $CD154^+$ T cells were isolated, as described above, and the number of $CD154^+$ cells after enrichment or the frequencies of $CD154^+$ cells after stimulation (1 μg/ml staphylococcal enterotoxin B, Sigma–Aldrich; anti-CD3/CD28 beads, Miltenyi Biotec) were analyzed by flow cytometry.

For Ag-processing experiments, T cells and APCs were separated from PBMCs via untouched isolation with the pan T cell isolation kit (Miltenyi Biotec) and depletion of $CD3^+$ T cells with anti-CD3 Microbeads (Miltenyi Biotec). Both fractions reached a purity >99%. CD3-depleted APCs were either loaded overnight with different Ags or left unloaded, and were subsequently fixed in 0.18% (v/v) paraformaldehyde in PBS/EDTA buffer for 5 min. A total of 1×10^7 cells was stimulated in an APC:T cell ratio of 1:1, and CD154-expressing cells were separated and analyzed by flow cytometry. The number of enriched $CD154^+$ cells was normalized to the control using nonfixed APCs plus Ags for stimulation.

Ab staining and flow cytometry analysis

PBMCs were stimulated and Ag-specific $CD4^+$ T cells were isolated, as described above. Depending on the experiment, enriched cells were stained in different combinations of the following mAbs (clone names in parantheses), according to manufacturer's protocols: CD14-PerCP, CD14-VioGreen (TÜK4), CD20-PerCP, CD20-VioGreen (LT20), CD3-PerCP (BW264/56), antibiotin-PE, antibiotin-VioBlue (Bio3-18E7), CD4-Vioblue, CD4-FITC, CD4-allophycocyanin, CD4-allophycocyanin-Vio770 (VIT4), CD27-FITC, CD27-allophycocyanin (M-T271), CD28-PE (15E8), CD95-PE (DX2), CD45RA-FITC, CD45RA-VioBlue (T6D11), CD45RO-allophycocyanin, and CD45RO-FITC (UCHL1) (all Miltenyi Biotec); CD11a-allophycocyanin (HI111), CCR7-AlexaFluor488 (MOPC-173), and CD45RA-PerCP.Cy5.5 (HI100) (all BioLegend); CD45RO-PE.Cy7 (UCHL-1; Becton Dickinson Biosciences); and CCR7-PE (150503; R&D Systems).

After fixation and permeabilization (Inside stain kit; Miltenyi Biotec), CD154 expression was stained with CD154-VioBlue, CD154-allophycocyanin, CD154-PE, and CD154-FITC (5C8; Miltenyi Biotec) alone, or in combination with different cytokines, as follows: TNF-α FITC (cA2), IFN-γ allophycocyanin, IFN-γ FITC, IFN-γ PE (45-15), IL-2 PE (N7.48A), IL-4 PE (7A3-3), IL-5 allophycocyanin (JES1-39D10), IL-10 allophycocyanin (B-T10), IL-13 PE (JES10-5A2.2), IL-17 FITC (CZ8-23G1) (all Miltenyi Biotec); IL-22 PE (142928; R&D Systems); IL-22 PerCP.Cy5.5 (22URTI; eBioscience); and IFN-γ PerCP.Cy5.5 (4S.B3; BioLegend).

Data were acquired on a MACSQuant analyzer, and MACSQuantify software (Miltenyi Biotec) was used for analysis.

Expansion and restimulation of Ag-specific T cell lines

Stimulation of PBMCs and isolation of $CD154^+$ cells were performed, as described above. Isolated $CD154^+$ cells were cultured with mitomycin C (Sigma–Aldrich)–treated autologous feeder cells in a ratio of 1:100 at a density of 2.5×10^6 cells/cm^2 in 48-well plates in X-Vivo15 (Lonza), supplemented with 5% (v/v) AB serum (Lonza), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 200 IU/ml IL-2 (Proleukin; Novartis). Cells were expanded for 14 d, culture medium was replenished, and cells were split, when needed.

For restimulation, autologous PBMCs were CD3 depleted with a purity >99% using anti-CD3 Microbeads (Miltenyi Biotec) and were cryopreserved until restimulation of expanded T cell lines. For restimulation, 5×10^5 expanded T cells were combined with thawed autologous CD3-depleted PBMCs in a ratio of 1:1 and stimulated with different Ags, in presence of 1 μg/ml CD28 functional grade pure Ab for 2 h plus additionally 4 h with 1 μg/ml brefeldin A (Sigma–Aldrich). After fixation and permeabilization, cells were stained intracellular for CD154 and cytokine expression.

For expansion of T cell lines from the naive or memory repertoire, $CD4^+$ T cells were isolated by negative selection using the naive $CD4^+$ T cell isolation kit or the memory $CD4^+$ T cell isolation kit (both Miltenyi Biotec), respectively. In brief, PBMCs from leukapheresis product were labeled with a mixture of biotin-conjugated Abs and antibiotin Microbeads for depletion of unwanted cell types. Sorted T cell subsets always reached a purity of >99%, as analyzed by flow cytometry. A total of 2×10^7 isolated naive or memory $CD4^+$ T cells was stimulated with autologous CD3-depleted PBMCs as APCs in a ratio of 1:1 and the different Ags. Enrichment, cultivation, and restimulation of reactive $CD154^+$ cells from both $CD4^+$ T cell subsets were performed, as described above.

Generation of Ag-specific T cell clones

Ag-specific T cell clones were generated by single-cell sorting from *A. fumigatus* or AdV-stimulated and magnetically pre-enriched $CD154^+CD4^+$ T cells using a FACS Aria III cell sorter (Becton Dickinson Biosciences). Single cells were sorted into 96-well round-bottom plates with 1×10^5/well irradiated (20 Gy) autologous feeder cells in 200 µl TexMACS medium (Miltenyi Biotec), supplemented with 15% AB serum, 2 µM 2-ME (Life Technologies), 2 mM glutamine, 300 IU/ml IL-2 (Proleukin), 100 IU/ml penicillin, 100 µg/ml streptomycin, and expanded polyclonal with 30 ng/ml anti-CD3 (OKT3; Miltenyi Biotec). After 7 d, 100 µl medium was replaced and 1×10^5 irradiated feeder cells were added. An additional 7 d later, cells were split into 96-well flat-bottom plates and further expanded. Restimulation was performed 4 wk after the single-cell sort by stimulating 5×10^5 expanded T cells/well with 5×10^5 autologous CD3-depleted PBMCs with or without Ag for 6 h. A total of 1 µg/ml brefeldin A was added for the last 4 h. Specificity of the expanded T cell clones was analyzed by intracellular cytokine staining.

Statistics

Statistical tests were performed with Prism software (GraphPad Software) using paired Student *t* test. For comparison of $CD154^+$ cell recovery with or without column staining (Supplemental Fig. 1A), unpaired Student *t* test was used. Pearson's correlation coefficient was used to calculate correlations. The *p* values <0.05 were considered statistically significant.

Results

$CD154^+$ enrichment allows quantitative capture and highly sensitive enumeration and characterization of Ag-specific $CD4^+$ T cells

CD154 is specifically expressed by all Ag-activated $CD4^+$ T cells, irrespective of defined peptide-MHC combinations and without any bias toward a certain cytokine-producing subset (24, 25). CD154-expressing $CD4^+$ T cells can readily be detected following 7-h stimulation of PBMCs with various viral (CMV, AdV), bacterial (tetanus toxoid), or fungal (*A. fumigatus*, *C. albicans*) recall Ags (Fig. 1A). However, it is also evident that, for certain Ags and/or donors, the frequencies of Ag-specific $CD4^+$ T cells are often <0.1%, which is in general the limit of detection for standard flow cytometry. In particular, at frequencies <0.1%, the resulting low total number of positive cells per sample (e.g., <100–1000 cells for sample sizes of 10^5–10^6 $CD4^+$ T cells) restricts the further dissection into specialized subpopulations. This limits the applicability of this method for many clinically and scientifically relevant situations.

To enable the analysis of Ag-reactive T cells from larger sample sizes (>10^6 cells), as required for rare cell detection, we magnetically pre-enriched the Ag-specific $CD154^+$ T cells before analysis. In this way, Ag-specific T cells from 10^6–10^9 PBMC could be rapidly selected and subsequently analyzed within short time. As shown in Fig. 1B, in total PBMCs stimulated with *A. fumigatus* lysate, ~70 $CD154^+$ $CD4^+$ T cells could be detected within 2×10^5 acquired events. In contrast, when 1×10^7 PBMCs were stimulated and the $CD154^+$ cells were enriched before the analysis, >2000 $CD154^+$ T cells could be detected by acquiring the entire positive fraction with only ~5×10^4 total events.

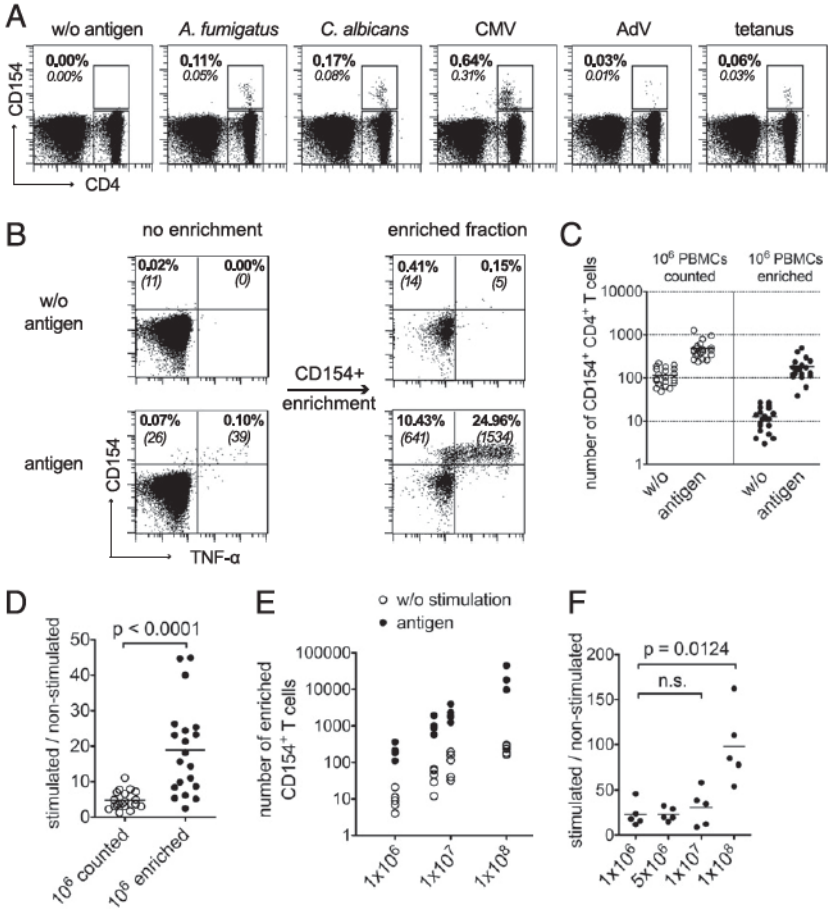

FIGURE 1. $CD154^+$ enrichment increases the sensitivity of detection of Ag-specific $CD4^+$ T cells. (**A**) Frequencies of $CD154^+$ cells among $CD4^+$ (bold) or total lymphocytes (italics) of one representative donor after stimulation of PBMCs with the indicated Ags. (**B**) Magnetic enrichment of $CD154^+$ T cells combined with intracellular cytokine staining. PBMCs were stimulated with *A. fumigatus* lysate or left unstimulated. CD154 expression was assessed among $CD4^+$ T cells without enrichment (*left plots*) and after $CD154^+$ enrichment (*right plots*). Numbers in brackets indicate the number of $CD154^+$ cells after acquiring 2×10^5 PBMCs (*left plots*) or obtained from 1×10^7 PBMCs after enrichment (*right plots*). (**C** and **D**) Comparison of the $CD154^+$ enrichment assay with standard flow counting using 1×10^6 starting PBMCs ($n = 20$; 4 independent experiments were performed). (C) $CD154^+$ cell numbers of nonstimulated or *A. fumigatus*–stimulated samples. To optimize the detection and quantification of $CD154^+$ events among $CD3^+CD4^+$ T cells, cell aggregates (scatter area versus scatter height) and non-T cell lineages ($CD14^+$, $CD20^+$, dump) were excluded. (D) Signal-to-noise ratio was calculated based on $CD154^+$ cell numbers in *A. fumigatus*–stimulated and nonstimulated PBMCs. Significance was determined using paired Student *t* test. (**E** and **F**) Detection limit of the $CD154^+$ enrichment assay ($n = 5$; 2 independent experiments were performed). (E) The total number of enriched $CD154^+$ cells from different starting cell numbers of *A. fumigatus*–stimulated or nonstimulated PBMCs is shown. (F) Signal-to-noise ratio was calculated based on $CD154^+$ cell numbers in *A. fumigatus*–stimulated and nonstimulated PBMCs. Significance was determined using paired Student *t* test.

By performing all staining and washing steps directly on the magnetic columns, the protocol was optimized to result in minimal cell loss during processing (Supplemental Fig. 1A), a minimal intra-assay variability, and a linear correlation between isolated target cells and the input cell number (10^6–10^8 PBMCs) (Supplemental Fig. 1B). This shows that the enrichment technology allows reproducible and quantitative assessment of rare CD154$^+$ T cells from large cell populations and enables to calculate back the frequency of Ag-specific CD4$^+$ T cells based on the number of enriched CD154$^+$ cells.

We further compared the background levels by acquiring the CD154$^+$ cells of 10^6 PBMCs with and without pre-enrichment. Using an optimized staining and analysis strategy (see Fig. 1 legend), the background without enrichment ranged between 50 and 200 CD154$^+$ cells per 10^6 counted PBMCs (Fig. 1C), that is, 0.02–0.07% among CD4$^+$ T cells, given an average frequency of 30% CD4$^+$ T cells in human PBMCs. In contrast, using the pre-enrichment strategy, the background was reduced by a factor of 10, whereas the number of CD154$^+$ target cells was only half reduced. Despite a strong donor-to-donor variation, this resulted in a considerably improved signal-to-noise ratio of ~5–50 fold with enrichment versus 2- to 10-fold without enrichment (Fig. 1D). Interestingly, at higher input cell numbers (up to 10^8), the number of isolated target cells increased linearly, whereas the proportion of background cells declined, resulting in an even superior signal-to-noise ratio at higher cell numbers (Fig. 1E, 1F).

These data show that magnetic pre-enrichment of CD154$^+$ T cells allows rapid, quantitative, and reproducible analysis of rare Ag-specific T cells from large sample sizes and results in a strongly improved signal-to-noise ratio. The detection limit ranges between 10^{-5} and 10^{-6} for 10^7–10^8 starting cells, but basically the sensitivity of the assay depends on the number of input cells.

CD154$^+$ enrichment specifically identifies Ag-reactive T cells

CD154 induction was dependent on MHC-peptide recognition, because an added HLA-DR–blocking Ab strongly inhibited CD154 upregulation on CD4$^+$ T cells, but had no effect on CD4$^+$ T cells activated by staphylococcal enterotoxin B or anti-CD3/CD28 (Supplemental Fig. 2A) nor on activated CD8$^+$ T cells (not depicted). Furthermore, the CD4$^+$ T cell responses against the Ag lysates but not against the pp65 peptide pool were prevented when APCs were fixed with formaldehyde before incubation with the Ags (Supplemental Fig. 2B), indicating that CD154 induction for native Ags is MHC class II and Ag processing dependent.

To further verify the specificity, the different recall Ag-activated CD154$^+$ T cells were magnetically isolated, expanded for 14 d, and subsequently restimulated with autologous APCs and the indicated Ags. The expanded T cells showed high reactivity toward the inducing Ag (30–60%), as demonstrated by the upregulation of CD154 and expression of cytokines (Fig. 2A), whereas reactivity against irrelevant Ags was at background level.

In addition, T cell clones were generated via single-cell FACS sorting of CD154$^+$ cells and expansion with anti-CD3 and IL-2. The cloning efficiency of the sorted CD154$^+$ cells was ~50% in each case (data not shown). Upon restimulation, 89% (107 of 120 clones) and 92% (110 of 120 clones) of initially *A. fumigatus* and AdV-stimulated clones, respectively, produced cytokines in an Ag-specific manner (Fig. 2B).

These data confirm the high specificity of the CD154$^+$ enrichment assay and demonstrate that specific T cell lines or clones can rapidly be generated for all tested Ags.

CD154$^+$ enrichment reveals pathogen-related functional heterogeneity of Ag-specific CD4$^+$ T cell responses

After applying ARTE for different recall Ags, the frequencies of Ag-specific T cells were calculated from the total number of

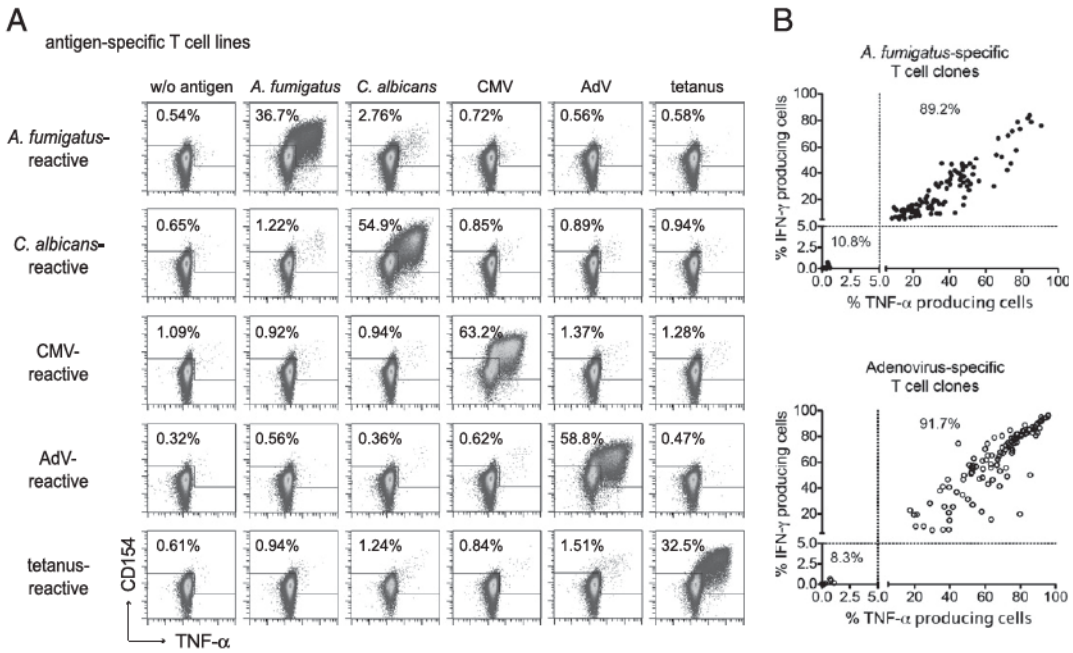

FIGURE 2. Specificity of expanded CD154$^+$ T cells. (**A**) PBMCs were stimulated with the indicated recall Ags. CD154$^+$ cells were isolated and subsequently expanded for 14 d with IL-2 and autologous feeder cells. Expanded cell lines were restimulated in presence of autologous APCs with and without Ags, as indicated, and reactive CD4$^+$ T cells were determined by CD154 and TNF-α expression. Representative dot plot examples of one donor of five with percentage of reactive cells among CD4$^+$ lymphocytes are shown. (**B**) T cell clones were generated from single CD154$^+$CD4$^+$ cells sorted by FACS from *A. fumigatus* lysate or AdV peptide pool-stimulated PBMCs. Cytokine expression was analyzed by intracellular staining upon Ag-specific restimulation. Dashed lines indicate the cutoff value of >5% cytokine producers. A total of 89.2% (107 of 120 clones) and 91.7% (110 of 120 clones) of the clones was specific for *A. fumigatus* and AdV, respectively.

$CD154^+$ cells obtained after enrichment relative to the total number of $CD4^+$ cells applied on the column. The frequency range for the different recall Ags was between 1 cell within 10^4–10^2 $CD4^+$ T cells, depending on the pathogen and the immune status of the donor (Fig. 3B, first diagram). The quality of an immune response is not only affected by the frequency or absolute number of the Ag-specific T cells, but also by their functional capacity, such as cytokine production. Dissection of pathogen-specific T cell responses into different functional T cell subsets therefore gives important insights into the induction of different functional capacities in vivo. A highlight of our method is that it allows the simultaneous assessment of the functional capacity of Ag-reactive T cells by multiparameter flow cytometric analysis, without long-term in vitro cultivation, thus directly reflecting the in vivo situation of the Ag-reactive T cells. Therefore, we used the $CD154^+$ preselection to analyze the cytokine profiles of Ag-specific T cells reactive against the various recall Ags at high resolution.

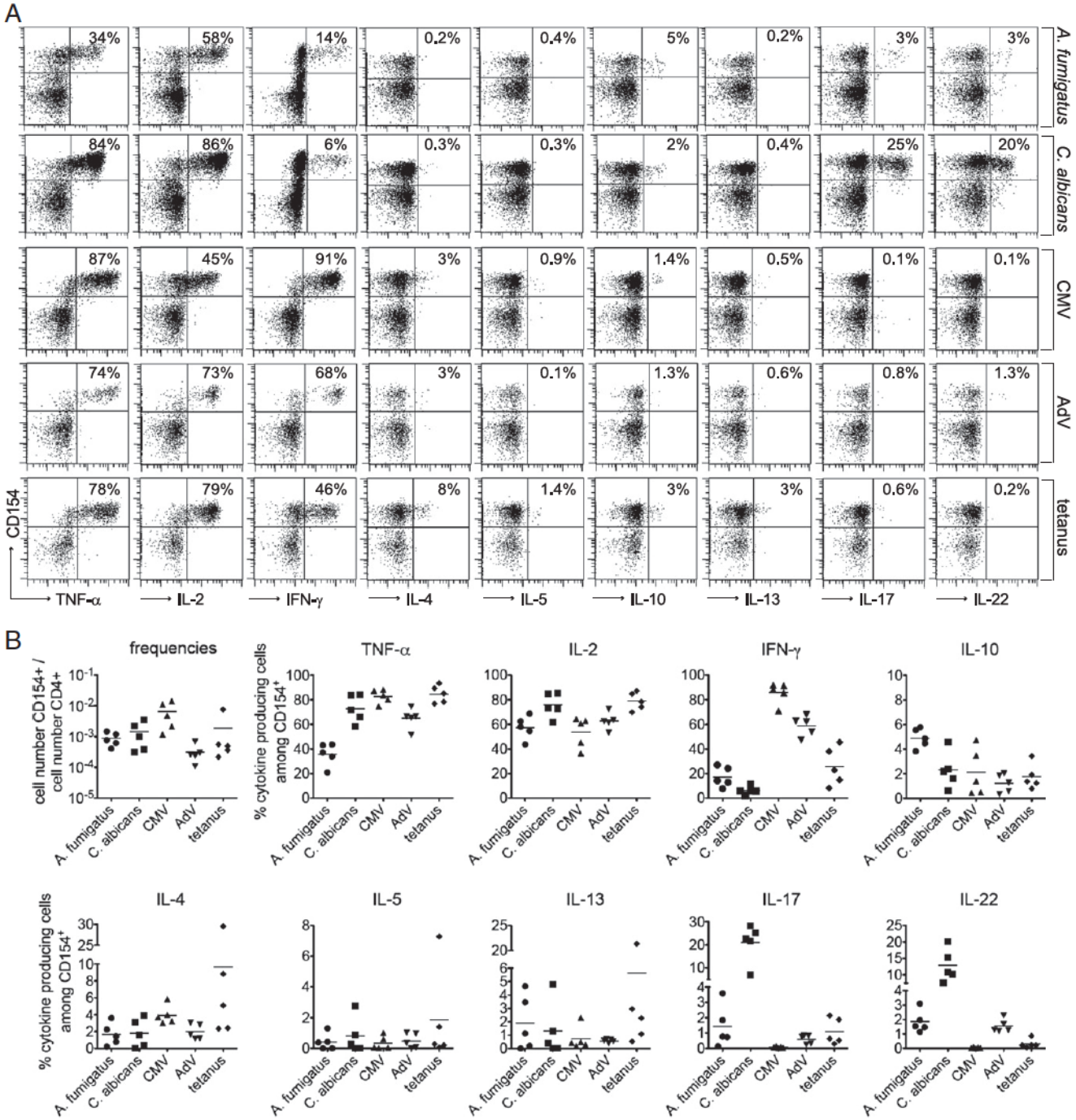

FIGURE 3. Characterization of small cytokine-producing subsets within the total Ag-specific T cell pool. (**A** and **B**) Flow cytometric ex vivo analysis of cytokine-expressing subsets within Ag-specific $CD4^+$ T cell responses. A total of 1×10^7 PBMCs was stimulated with the indicated Ags, and Ag-specific T cells were analyzed for cytokine expression using the $CD154^+$ enrichment assay. Cells were gated on $CD4^+$ lymphocytes, and percentages of cytokine-expressing cells among $CD154^+$ T cells are shown. (A) Representative dot plot examples and (B) statistical analysis from several donors with indicated mean values ($n = 5$; two independent experiments were performed). Frequencies of Ag-specific T cells (first diagram) were calculated from the total number of $CD154^+$ cells obtained after enrichment normalized to the total number of $CD4^+$ cells applied on the column.

The power of our method is illustrated in Fig. 3, as follows: cytokines, which are not restricted to a certain T cell lineage, for example, IL-2 or TNF-α, occurred at high frequencies (up to 85%) in the Th cell responses against all analyzed Ags. However, most other cytokines, including the lineage-defining cytokines IFN-γ, IL-4, IL-17, and IL-22, which are important parameters for the classification of Th cell responses into Th1, Th2, Th17, or Th22 subsets, showed much higher variability and represented often only 1–10% of the total Ag-specific T cell pool, that is, 0.0001–0.1% for the Ags analyzed in this work (Fig. 3). As depicted in Fig. 3A, the magnetic preselection allows the collection of a number of Ag-reactive T cells, high enough to enable the precise identification of small cytokine-producing subsets, down to 1–10% within the Ag-reactive $CD4^+$ T cell pool (Supplemental Fig. 3) (28).

Th cell responses against certain pathogens are often classified according to a certain dominant cytokine expression. For example, viral Ags have been shown to induce Th1 responses, extracellular pathogens, Th2 responses, and fungi Th17 responses. However, besides these dominant signatures, our refined analysis revealed a much higher heterogeneity in the functional cytokine repertoire of Ag-specific T cells. Consistent with previous data (29), the response against *C. albicans* was of a clear Th17 phenotype, with high levels of IL-17 and IL-22 producers (10–30%), but we also detected small populations of Th1 (IFN-γ)- and Th2 (IL-4, IL-5, IL-13)-type cells that mainly did not coproduce IL-17 or IL-22 (data not shown). In contrast, in the T cell response against another fungal pathogen, *A. fumigatus*, the Th1 cytokine IFN-γ was dominant (10–30%), but also distinct populations of non-Th1 cytokines, such as IL-10 (4–6%), IL-17, and IL-22 (1–5%), as well as Th2 cytokines (IL-4 and IL-13, 1–5%), were present in most donors.

In contrast, the response against the vaccine Ag tetanus was less dominated by a single signature with intermediate levels of Th1 cells (IFN-γ, 10–40%) and quite variable frequencies of Th2 cells (IL-4, 2–30%; IL-5, 1–6%; IL-13, 1–20%). In some donors, we also observed a small population of IL-17 producers, but almost no IL-22 producers. However, even within the highly Th1-polarized antiviral immune responses against CMV and AdV, we found small differences with regard to non-Th1 cytokines. For example, we detected clear populations of IL-4 and IL-10 producers against CMV, whereas AdV-specific T cells also contained a small population of IL-17 and IL-22 producers that were completely absent in the response to CMV. Interestingly, despite this heterogeneity in the cytokine patterns against different pathogens, the distribution on the different T cell subsets was quite similar for different donors (Fig. 3B), indicating indeed the existence of pathogen-related cytokine signatures. Because most of the cytokines are restricted to small subpopulations, we use the term microsignatures to differentiate them from the classification according to the dominant Th cell signatures.

Taken together, to our knowledge, our method enables for the first time to directly visualize the total ex vivo functional repertoire of complex pathogen-specific T cell responses on a single-cell level at high resolution. The refined subpopulation analysis reveals that, in addition to the major cytokine-producing subsets, the detection of rare cytokine-producing T cells allows to identify an unexpected broad complexity within the pathogen-specific $CD4^+$ T cell responses.

ARTE unmasks the naive T cell repertoire, and enables the direct visualization of autoreactive T cells even from healthy donors

Until now, the cytometric analysis of Ag-reactive T cells using cytokines or activation markers as a readout was mainly restricted to the analysis of the memory T cell pool because the frequency of naive T cells was below the level of detection. The high sensitivity of our method prompted us to extend our study to the analysis of Ag-specific $CD4^+$ T cell responses in the naive T cell repertoire. We therefore investigated the human T cell repertoire reactive against auto- and neo-Ags, which have to date escaped direct quantitative and qualitative characterization. We applied ARTE to analyze the $CD4^+$ T cell responses of healthy donors against peptide pools of six different auto (MOG, GAD)-, tumor-associated (NY-ESO, WT-1), or neo-Ags (KLH, HIV Gag), and MP65, a major *C. albicans* Ag, as a positive control. Without enrichment, no $CD154^+$ T cells against the auto- and neo-Ags were detectable within 5×10^5 acquired PBMCs, whereas MP65-reactive T cells could immediately be detected with a frequency of ~1 in 10^4 $CD4^+$ T cells (range 10^{-3}–10^{-5}) (Fig. 4A). However, following the enrichment from 10^8 stimulated PBMCs, reactive T cells against all Ags were clearly detected in all donors tested, with frequencies ranging from $<10^{-6}$ to 10^{-4} (Fig. 4A, 4B). The frequencies of T cells differed between the various Ags, but, for a particular Ag, rather small donor-to-donor variations were observed. Surprisingly, we found similar frequencies (10^{-5} to 10^{-4}) against the auto-Ags, MOG and GAD, and the neo-Ags, KLH and HIV Gag. However, the frequencies against the tumor-associated Ags NY-ESO and WT-1 were ~10-fold lower (10^{-6}–10^{-5}). Further phenotypic analysis of the enriched $CD154^+$ cells revealed that a large fraction, but not all of the T cells reactive against the auto-, tumor-associated, or neo-Ags indeed have a naive phenotype (40–60%, $CD45RO^-CCR7^+$), whereas *C. albicans* MP65-specific $CD4^+$ T cells were mainly memory cells (80–90%, Fig. 4C, 4D). These data demonstrate that the $CD154^+$ enrichment assay can be used to monitor the human naive Ag-reactive T cell repertoire. The data also indicate that even in healthy donors a significant fraction of the self-reactive repertoire has a clear memory phenotype, which suggests a role for cross-reactivity against external Ags for priming of autoreactive T cells even in a healthy individual.

Generation of Ag-specific T cell lines following $CD154^+$ enrichment from the naive $CD4^+$ T cell compartment

To confirm the specificity of the $CD4^+$ T cells reactive against auto-, tumor-associated, and neo-Ags within the naive, as well as in memory compartment, reactive cells were enriched from highly purified naive and memory $CD4^+$ T cells (Supplemental Fig. 4A, 4B), expanded for 14 d, and tested for specificity by Ag restimulation. As shown in Fig. 5, expanded T cells from the naive as well as from the memory population specifically reacted against the inducing Ag. Interestingly, for the auto-, tumor-associated, and neo-Ags, but not for the recall Ag MP65, the frequency of specific T cells within the expanded T cell lines was higher for T cell lines generated from naive T cells versus memory-derived cell lines. This may indicate lower affinity or reduced proliferative potential of self-reactive T cells within the memory pool. However, these data clearly confirm our ex vivo phenotypic observations and demonstrate the applicability of ARTE for the direct ex vivo detection, enumeration, and characterization of Ag-specific T cell populations, undetectable by standard assays.

Discussion

The Ag-induced expression of CD154 has been described as a versatile tool for the direct ex vivo detection of $CD4^+$ T cells specific for any pathogen or Ag of interest (24–26). We employed its high specificity in combination with a magnetic enrichment step of rare $CD154^+$ cells from large cell samples. In this way, the sensitivity of detection is mainly restricted by the number of input

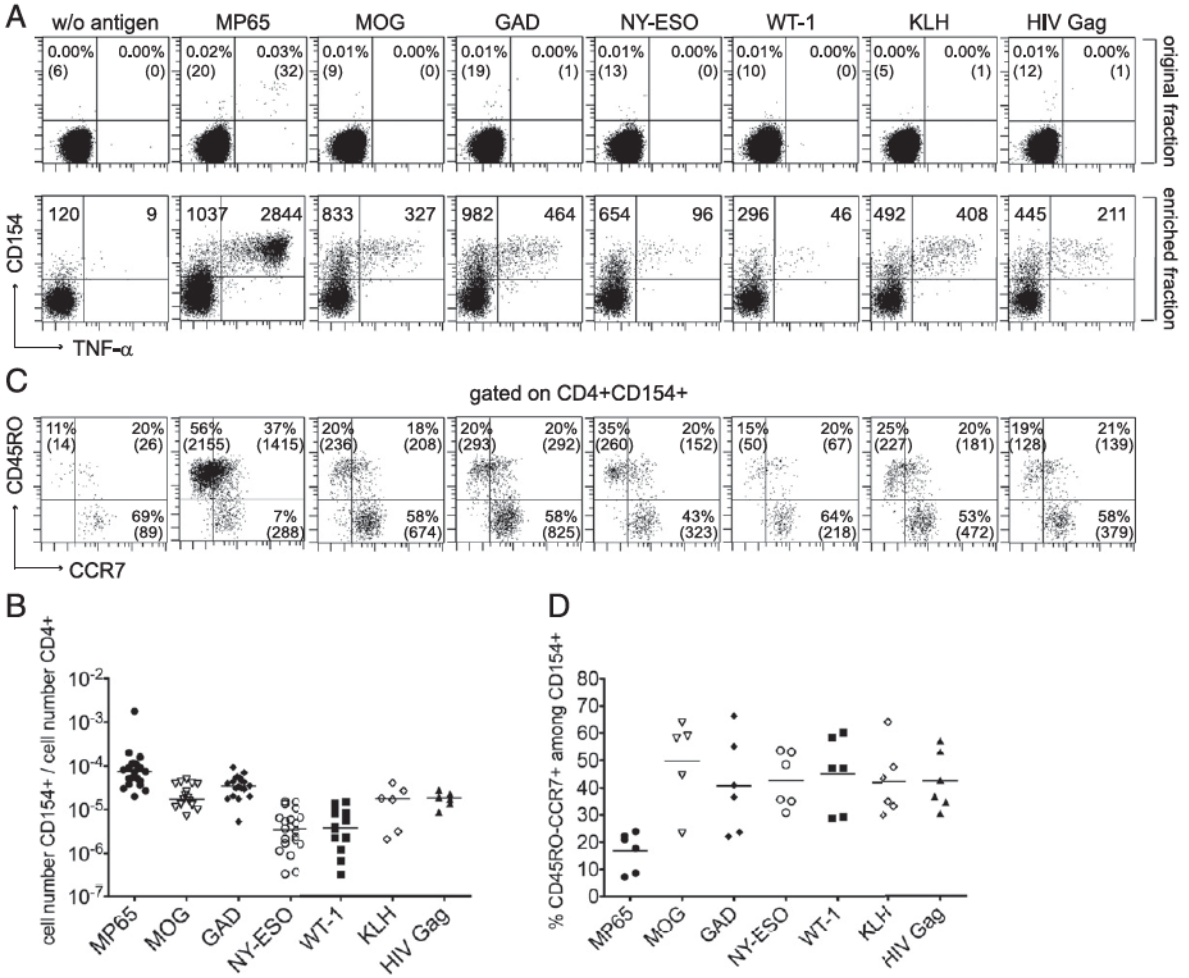

FIGURE 4. Enumeration and characterization of $CD4^+$ T cells reactive against auto- or neo-Ags. (**A**) A total of 1×10^8 PBMCs was stimulated as indicated, and $CD154^+$ expression among $CD4^+$ T cells was analyzed without enrichment (*upper plots*) and after performing the $CD154^+$ enrichment assay (*lower plots*). Indicated are percentages of $CD154^+$ cells among $CD4^+$ and number of $CD154^+$ cells after acquiring 5×10^5 PBMCs (*upper plots*) or obtained from 1×10^8 PBMCs after enrichment (*lower plots*). (**B**) Enumeration of rare Ag-specific $CD4^+$ T cells in several donors using the $CD154^+$ enrichment assay. The total number of enriched $CD154^+$ T cells was determined using a single, live, nondump, $CD3^+CD4^+$ gating strategy, and background enriched from the nonstimulated control was subtracted. Depicted is the total number of $CD154^+$ cells obtained after enrichment normalized to the total number of $CD4^+$ cells applied on the column (MP65, $n = 21$; GAD, NY-ESO, $n = 19$; MOG, WT-1, $n = 16$; KLH, HIV Gag, $n = 6$). (**C** and **D**) Enriched $CD154^+$ cells were ex vivo analyzed for phenotypic surface markers CD45RO and CCR7. Cells are gated on $CD4^+CD154^+$ lymphocytes. (C) Representative dot plot examples from one donor with percentages of cells among $CD154^+$ cells and cell count (in brackets) and (D) statistical analysis for percentage of $CD45RO^-CCR7^+$ cells among the total number of $CD154^+$ cells ($n = 6$; two independent experiments were performed). Background enriched from the nonstimulated control was subtracted.

cells, that is, for 10^7–10^8 PBMCs we estimated a sensitivity of 10^{-5}–10^{-6}. This allowed us to directly access extremely rare populations such as Ag-specific T cells within the naive $CD4^+$ T cell repertoire as well as to dissect the Ag-specific memory repertoire at high resolution.

High resolution, that is, the characterization of minor subpopulations with high statistical precision, requires the collection of sufficient target events, allowing a proper statistical analysis. For example, to estimate a 10% subset of a population with a coefficient of variation <10%, at least 1000 $CD154^+$ T cells have to be acquired (Supplemental Fig. 3) (28). The magnetic preselection allowed the collection of a number of Ag-reactive T cells, sufficient for the identification of small cytokine-producing subsets, down to 1–10% within the total reactive $CD4^+$ T cell pool. The power of magnetic pre-enrichment has already been demonstrated for various rare cell populations (30–32) and has been employed for MHC-multimers to identify Ag-specific T cells even in the naive repertoire (1, 18–20). However, the method we describe in this work has several advantages over the MHC-multimer technology, which is restricted to a set of exactly defined peptide-MHC combinations. Therefore, the $CD154^+$ enrichment technology fills an apparent gap in the Ag-specific T cell analysis tool box, now also providing ex vivo access to the functional $CD4^+$ T cell pool with a sensitivity comparable to the MHC-multimer enrichment approach, but without the need for in vitro expansion or restriction to a certain Ag or MHC allele.

We used this method to dissect the $CD4^+$ T cell response against various pathogens into small but functionally distinct subpopulations. Many analyses restrict the characterization of pathogen-specific T cells to rather abundant cytokines such as IL-2, TNF-α, or IFN-γ, due to the limited access to Ag-specific T cell numbers. In this study, we show that many functionally important cytokines, such as IL-4, IL-5, IL-10, and IL-13, and for many pathogens, also IL-17 and IL-22 are produced only by ~1–10% of the total Ag-specific T cell pool and therefore may represent only 0.01–0.001% of the total $CD4^+$ T cell compartment. These data dem-

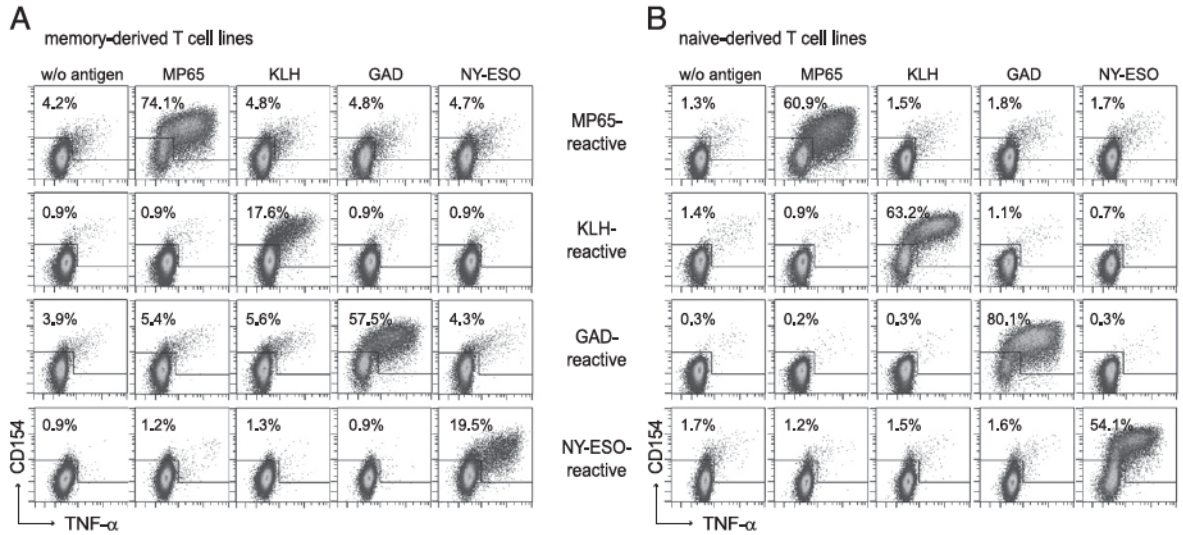

FIGURE 5. CD154$^+$ enrichment allows generation of Ag-specific T cell lines from the naive CD4$^+$ T cell repertoire. A total of 2×10^7 purified memory (**A**) or naive (**B**) CD4$^+$ T cells was stimulated with CD3-depleted APCs and the indicated Ags. Enriched CD154$^+$ cells were expanded for 14 d with IL-2 and autologous feeder cells. Expanded cell lines were restimulated in presence of autologous APCs with and without Ags, as indicated, and reactive CD4$^+$ T cells were determined by CD154 and TNF-α expression. Representative dot plot examples of one donor of three with percentage of reactive cells among total CD4$^+$ are shown.

onstrate that the rather simplified classification of an immune response into Th1, Th2, or Th17 according to the few dominant lineage-defining cytokines can be refined by identification of the underlying cytokine microsignatures. Indeed, our analysis of the cytokine-producing subsets revealed an unexpected broad functional heterogeneity of T cells reacting against the different pathogens. Interestingly, the responses against a given pathogen were remarkably similar between different donors, indicating indeed the existence of pathogen-related cytokine signatures that can be identified only via high-resolution analysis.

One interesting finding was that the two fungal pathogens *A. fumigatus* and *C. albicans* elicit completely different patterns of Th cell responses. Although Th17 responses are often described as prototypic for antifungal immunity, we show that only *C. albicans*–reactive T cells produce mainly IL-17 and IL-22, whereas the response against *A. fumigatus* was rather dominated by IFN-γ–producing Th1 cells and also contained a small fraction of IL-10 producers. Interestingly, also a small fraction of *A. fumigatus*–specific Th17/22 cells was consistently present throughout all donors. The Th17 versus Th1 signatures might reflect the selective capability of *C. albicans* to elicit Th17 responses in vivo and in vitro, as it has recently been described (29, 33), or different infection routes of the two pathogens, that is, the lung for *A. fumigatus* versus gut and skin epithelia for *C. albicans*. However, it will be interesting to further analyze the origin of the *A. fumigatus*–reactive Th17 versus Th1 subset and to define which role the two subsets possess in the various antifungal immune responses, that is, allergies or invasive infections.

Also, in the antiviral CD4$^+$ T cell responses, which are clearly dominated by a Th1 cytokine profile, we found a second, heterogeneous response of non-Th1 cytokines, specifically associated with a certain virus. In particular, IL-10 and IL-4 were found at low level against both viruses, but were more pronounced in response to CMV, whereas IL-22 and at lower level also IL-17 producers, which were consistently present in the AdV-specific immune response, were absent against CMV (<0.1%). This indicates that, besides the major Th subset-inducing capacity of certain classes of pathogens, for example, Th1 for viruses, different pathogens have additional capabilities to induce distinct cytokine microsignatures not necessarily related to the dominant signature. It will be interesting to identify the critical factors responsible for the induction of these cytokine microsignatures that all together constitute functional T cell immunity, such as the nature of the pathogen-associated signals, Ag dose, the site of Ag exposure, or the preparation of vaccine adjuvants, as well as potential cross-reactivity between species with different cytokine-inducing capabilities. Indeed, the importance of multiparameter characterization of the Ag-specific repertoire, for example, for prediction of immune protection, has been highlighted before by many studies (4–6). Thus, the possibility to directly visualize now in detail the broad heterogeneity of Ag-specific CD4$^+$ T cell responses at high resolution will be an important tool to identify the underlying principles of subset heterogeneity and their use for diagnostic assays, for example, during immunotherapies.

Another important application of our technology was the analysis of Ag-specific T cells in the naive T cell compartment, which could to date not be directly assessed without restriction to selected peptides. Knowledge about Ag specificity in the naive repertoire may allow to predict the success of immunomodulation strategies, such as vaccination and tolerization, or to assess the risk for the development of immunopathologies. To our knowledge, the high sensitivity of the CD154$^+$ enrichment assay allowed us for the first time to visualize the full repertoire of CD4$^+$ T cells reactive against various neo-, auto-, or tumor-associated Ags directly from PBMCs of healthy donors. We observed frequencies of reactive cells against these Ags in a range of $<10^{-6}$–10^{-4}, which is quite similar to the frequencies of naive CD4$^+$ T cells specific for other naturally processed Ags determined by the T cell library approach (2). However, recent studies in humans, using MHC-multimer enrichment, detected frequencies of Ag-specific naive CD8$^+$ and CD4$^+$ T cells of 0.3–3.6 (1) and 0.2–10 (18) per million cells, respectively. The higher frequencies found in our study are most probably due to the fact that we did not determine frequencies of T cells specific for a single epitope. The peptide pools used in this study consist of overlapping 15-mer peptides (11-aa overlap), covering the complete protein sequence and all possible T cell epitopes (34). Therefore, the total frequency of Ag-specific T cells most likely reflects responses to multiple epitopes.

Surprisingly, although a large number of the neo-, auto-, or tumor Ag-specific T cells was indeed in the naive state, as expected for healthy individuals, the reactive cells always contained a significant fraction of memory T cells. These are truly Ag-specific T cells because enriched cells from both T cell compartments gave rise to highly specific T cell lines following in vitro expansion. In healthy donors, memory T cells against auto-Ags and in particular against the neo-Ag KLH are most probably the result of cross-reactivity to external Ags. Accordingly, we observed a tendency that T cell lines against the neo- and auto-Ags were less efficiently generated from memory versus naive derived T cells, whereas pathogen-reactive T cell lines could efficiently be generated from memory T cells. This may be explained by lower affinity of T cells reactive against the cross-reactive versus the original priming Ag. Alternatively, this could be due to a reduced proliferative potential that may indicate an anergic state of the autoreactive memory T cells, although this would not apply for T cells reactive against neo-Ags like KLH or HIV gag. However, the contribution of cross-reactive memory T cells against neo- and auto-Ags to immune protection or the development of autoimmunity requires further investigation. Interestingly, the number of reactive cells against a certain neo- or auto-Ag was relatively constant between different donors, but differed between the various Ags, as described before for $CD8^+$ T cells (1). Specifically, T cells reactive against the tumor-associated Ags NY-ESO and WT-1 were ~10-fold less frequent, but contained similar proportions of naive and memory T cells. Whether these differences result from differences in thymic negative selection or peripheral depletion or simply result from a lower number of available T cell epitopes remains to be analyzed. However, in the murine model, it has been shown that the size of a certain naive $CD4^+$ T cell population is strongly affected by negative thymic selection with a stronger thymic deletion of smaller naive T cell populations (35). Furthermore, a correlation between naive precursor frequencies and the size and TCR diversity of the memory responses to different peptides has been reported (19, 35, 36). Because NY-ESO and WT-1 are used for antitumor vaccination, it would be interesting to study whether the frequency of precursors predicts the outcome of vaccination, as it has, for example, recently been shown for anthrax vaccination (18).

In summary, the $CD154^+$ enrichment technology allows the visualization and quantification of functional Ag-specific T cells at unprecedented sensitivity, providing even access to the naive $CD4^+$ T cell repertoire. Our results highlight the importance of dissecting the total Ag-reactive T cell pool into small, but functionally relevant subsets, giving a more precise picture of the functional T cell repertoire. The method allows quantification of various parameters with potential diagnostic or prognostic relevance within one single, short, and sensitive assay and can in principle be extended to any pathogen or Ag of interest. Because the absolute cell numbers available for a single analysis are low or require sample sizes that are difficult to obtain routinely from patients, as many parameters as possible should be measured in parallel. Therefore, recent developments, which have dramatically increased the number of parameters for cytometric analysis, will optimally synergize with the Ag-reactive T cell enrichment technology described in this work (37). The accurate and sensitive assessment of Th cell immunity provides new, exciting possibilities for basic and clinical research and represents a powerful diagnostic tool for infections, autoimmunity, and allergies.

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Disclosures

P.B. and M.A. are employees of Miltenyi Biotec. A.S. works as a consultant for Miltenyi Biotec. Miltenyi Biotec provides materials and reagents suitable for performing the assays described in this paper and has filed IP rights for commercial use of the described method. The other authors have no financial conflicts of interest.

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Supplementary Figure 1 Bacher et al.

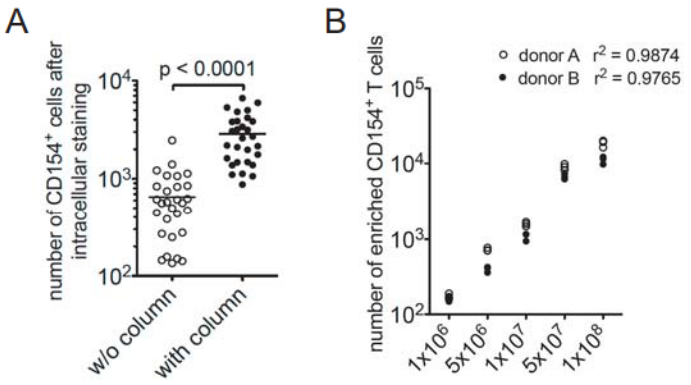

Figure S1. $CD154^+$ enrichment assay allows accurate and reproducible quantification of rare antigen-specific T cells. (**A**) Performing intracellular staining directly on the magnetic columns strongly increases cell recovery. $1x10^7$ PBMCs were stimulated with *A. fumigatus* lysate and the recovery range of $CD154^+$ cells following intracellular staining performed either directly on the magnetic column or after elution from the column was analyzed (n = 30). Horizontal bars indicate mean values and significance was determined using unpaired Student´s *t*-test. (**B**) Intra-assay variability of the recovered $CD154^+$ cell numbers. Different PBMC starting cell numbers from two donors were stimulated in triplicates with *A. fumigatus* lysate and recovered $CD154^+$ cell numbers were analyzed after performing the $CD154^+$ enrichment assay. Pearson´s correlation coefficient was used to calculate correlations ($p < 0.0001$).

Supplementary Figure 2 Bacher et al.

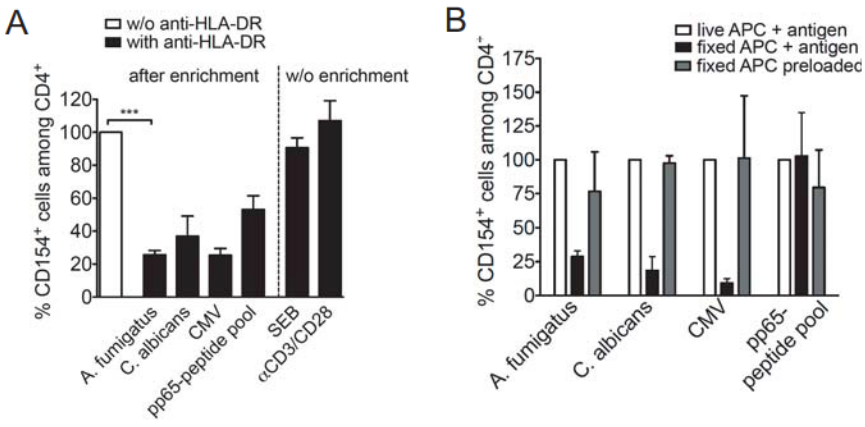

Figure S2. Specific induction of $CD154^+$ expression following antigen stimulation. (**A**) Inhibition of the CD154 induction by HLA-DR blockade. PBMCs of CMV pp65-tetramer positive donors were stimulated with the indicated antigens in the presence or absence of anti-HLA-DR and the number of $CD154^+$ T cells after enrichment (left) or the frequencies of $CD154^+$ T cells without enrichment (right) were analyzed. Bars show mean percentages and SEM from 4 donors referring to samples without anti-HLA-DR. Two independent experiments were performed. (**B**) CD154 induction is antigen-processing dependent. Stimulation of isolated $CD3^+$ T cells was performed under the indicated conditions and the number of $CD154^+$ T cells after enrichment was analyzed. Bars indicate percentages of $CD154^+$ cell numbers under the various conditions relative to the stimulation with live APCs + antigen from 3 different donors.

Supplementary Figure 3 Bacher et al.

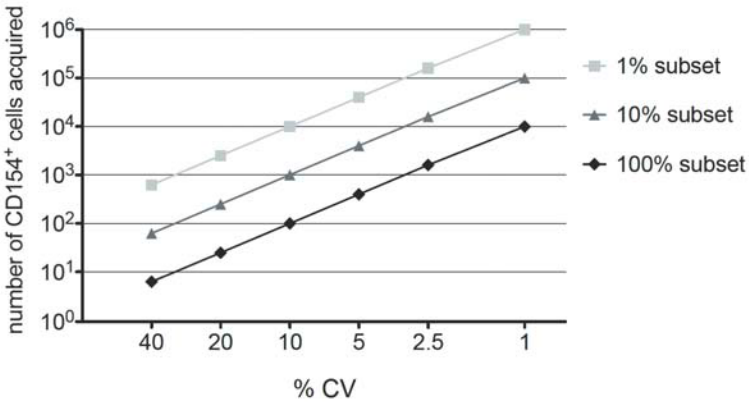

Figure S3. The precision of rare cell analysis is dependent on the number of acquired target cells. To describe the precision of flow cytometry data, the coefficient of variance (CV) can be calculated from the variance and the standard deviation (SD). For rare cell analysis, the approximations SD = $\sqrt{r}$ and CV % = $100/\sqrt{r}$ can be used, where r is the number of positive events (28).

Using this approximation, the CV of a certain cytokine producing subset is illustrated, depending on the number of acquired $CD154^+$ target cells. For example, to obtain a CV of 10%, 100 $CD154^+$ cells must be acquired. If the cytokine producing cells are a subpopulation of 10% among all $CD154^+$ cells, 1000 $CD154^+$ cells need to be acquired.

Supplementary Figure 4 Bacher et al.

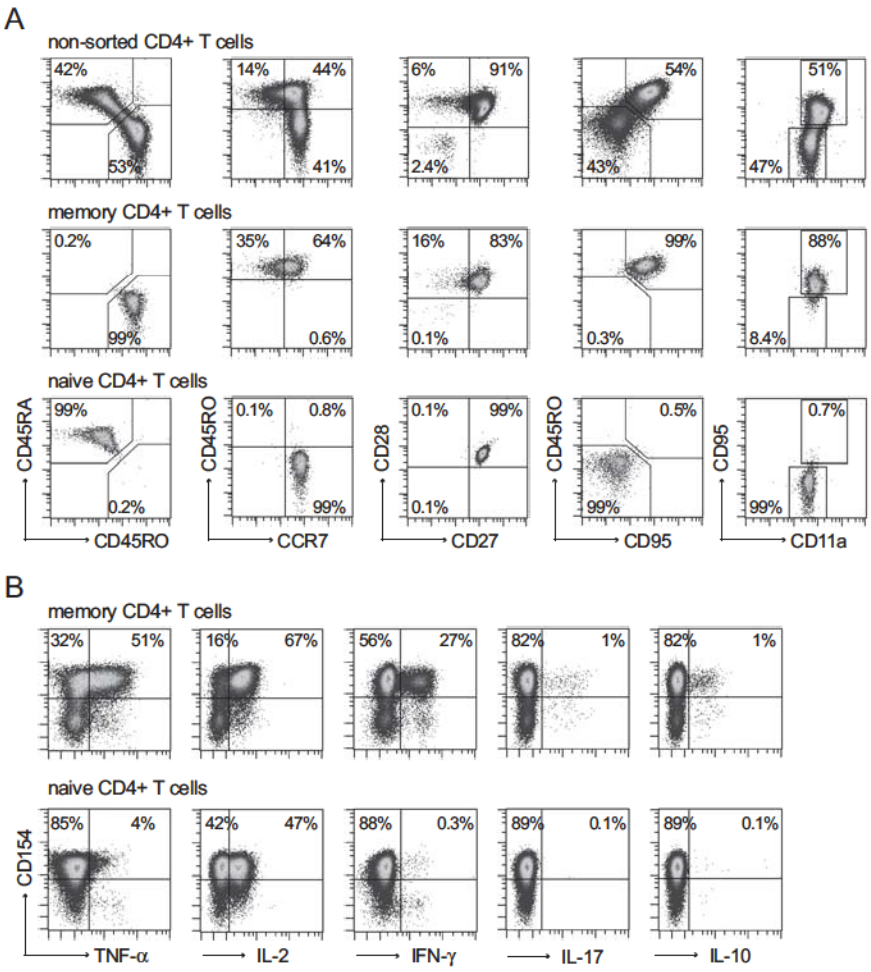

Figure S4. Phenotypic and functional characterization of isolated naive and memory $CD4^+$ T cells. (**A**) Phenotypic characteristics of non-sorted $CD4^+$ T cells and isolated memory and naive $CD4^+$ T cells subsets. (**B**) Cytokine expression by T cell subsets: Isolated memory and naive $CD4^+$ T cells were stimulated with PMA/Ionomycin and analyzed for intracellular $CD154^+$ expression and cytokine production. Data are representative of three independent experiments.

3.3 Manuscript III

"Antigen-specific Expansion of Human Regulatory T Cells as a Major Tolerance Mechanism against Mucosal Fungi."

Bacher P, Kniemeyer O, Schönbrunn A, Sawitzki B, Assenmacher M, Rietschel E, Steinbach A, Cornely OA, Brakhage AA, Thiel A, Scheffold A.

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Antigen-specific expansion of human regulatory T cells as a major tolerance mechanism against mucosal fungi

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Foxp3$^+$ regulatory T cells (Treg) have a central role for keeping the balance between pro- and anti-inflammatory immune responses against chronically encountered antigens at mucosal sites. However, their antigen specificity especially in humans is largely unknown. Here we used a sensitive enrichment technology for antigen-reactive T cells to directly compare the conventional vs. regulatory CD4$^+$ T-cell response directed against two ubiquitous mucosal fungi, *Aspergillus fumigatus* and *Candida albicans*. In healthy humans, fungus-specific CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Treg are strongly expanded in peripheral blood and possess phenotypic, epigenetic and functional features of thymus-derived Treg. Intriguingly, for *A. fumigatus*, the strong Treg response contrasts with minimal conventional T-cell memory, indicating selective Treg expansion as an effective mechanism to prevent inappropriate immune activation in healthy individuals. By contrast, in subjects with *A. fumigatus* allergies, specific Th2 cells were strongly expanded despite the presence of specific Treg. Taken together, we demonstrate a largely expanded Treg population specific for mucosal fungi as part of the physiological human T-cell repertoire and identify a unique capacity of *A. fumigatus* to selectively generate Treg responses as a potentially important mechanism for the prevention of allergic reactions.

INTRODUCTION

The mucosal immune system is constantly confronted with the challenge to combat a vast number of pathogens from the environment without causing collateral tissue damage due to uncontrolled or inappropriate immune reactions. There is substantial evidence from animal experiments that regulatory T cells (Treg) have an important role in maintaining tolerance against commensals or inhaled antigens encountered at mucosal sites.[1–6] However, the antigen specificity of this protective Treg population in particular in humans remained so far elusive due to the high number of potential targets and a lack of specific and sensitive technologies for the direct quantitative and qualitative analysis of Treg with a defined antigen specificity.[7]

Fungi represent a prototypic example for mucosally encountered environmental pathogens and/or commensal microbiota whose importance has been underestimated for many years.[8] Humans continuously inhale several thousands of airborne fungal spores per day.[9,10] Other fungi such as *Candida albicans* belong to the normal mucosal microflora of humans.[11] In immunocompromised patients, fungi such as *Aspergillus fumigatus* and *Candida albicans* can cause life-threatening invasive infections.[12] In addition, inhaled airborne fungi can cause allergic hypersensitivity especially in patients with respiratory disorders, such as severe asthma or cystic fibrosis (CF). These patients frequently suffer from severe Th2-driven allergic reactions and allergic bronchopulmonary aspergillosis (ABPA).[13]

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Strikingly, despite daily contact with fungal antigens, immunocompetent persons are highly resistant to severe fungal infections as well as hypersensitivity reactions.[14] This implicates the existence of efficient protective immune responses as well as regulatory mechanisms that are able to prevent inappropriate immune reactions.[15] It has been shown that fungal components trigger efficient innate immune reactions and eventually also initiate adaptive immune responses mainly mediated by $CD4^+$ T cells.[16,17] We recently showed that fungus-reactive conventional memory $CD4^+$ T cells are indeed present in peripheral blood of all healthy donors[18] (and unpublished data), which most likely reflects the ubiquitous presence of fungal antigens in the environment. However, the cellular basis for anti-inflammatory or tolerogenic mechanisms, which avoid immunopathology such as chronic inflammation or allergies against fungi is less clear. We therefore analyzed Treg as well as conventional naive and memory $CD4^+$ T cells (Tcon) specific for the two major fungal pathogens *A. fumigatus* and *C. albicans* directly in blood of human subjects. This comparison was possible by using a new sensitive pre-enrichment technology for the direct detection of rare antigen-specific Tcon and Treg, according to the converse expression pattern of the activation markers CD154 and CD137.[18,19] Interestingly, we found that both fungi, but in particular *A. fumigatus,* elicited a strong Treg response in adult but not in cord blood. For *C. albicans*, the specific Treg response was accompanied by a parallel dominant memory Tcon (Tmem) formation. However, for *A. fumigatus*, the balance was strongly shifted toward Treg as the specific Tcon were composed of only a minimally expanded memory and a large naive compartment. Thus, Treg numbers even exceeded those of Tmem, resulting in a marked suppression of the anti-*A. fumigatus* Tcon response in *in vitro* assays. This presumably protective Treg dominance was abrogated in CF patients with *Aspergillus*-associated allergies due to a strong expansion of Th2-type Tmem. These data provide direct evidence for the preferential generation of an antigen-specific Treg response against a chronically encountered mucosal pathogen and suggest that subversion or bypassing of this mechanism contribute to the development of allergies.

RESULTS

High frequencies of fungus-specific memory Treg in peripheral blood of healthy donors

To better define the contribution of T cells to the delicate balance between pro- and anti-inflammatory immune responses against chronically exposed fungi, we performed a side-by-side comparison of human Tcon and Treg responses against *A. fumigatus* and *C. albicans*. Converse expression of CD137 and CD154 following short-term stimulation with fungal lysates enabled identification of activated forkhead box protein P3 (Foxp3) expressing Treg together with Tcon.[19]

Stimulation with *A. fumigatus* and *C. albicans* antigens induced overall low frequencies of $CD154^+$ or $CD137^+$ $CD4^+$ T cells (**Figure 1a** and **b**). Despite their low overall abundance, this corresponds to surprisingly high frequencies within the $CD25^+Foxp3^+$ population, which actually represents only 5–10% of the total $CD4^+$ T cells, i.e., 1.3% (range 0.06–3.4%) for *A. fumigatus* and 0.6% for *C. albicans* (range 0.02–2.6%) (**Figure 1c**). In contrast, the frequencies of fungus-reactive Tcon among total $CD4^+$ T cells were about 5- to 10-fold lower, and here the frequencies of *C. albicans*-reactive Tcon were significantly higher (0.27%; range 0.11–0.55%) than those of *A. fumigatus*-reactive Tcon (0.19%; range 0.06–0.37%).

For a more sensitive and detailed analysis, we used antigen-reactive T-cell enrichment (ARTE)[7,18] to enable the quantification and characterization of the few $CD154^+$ and $CD137^+$ cells from larger cell samples ($\geq 1 \times 10^7$ PBMC) with high sensitivity and specificity (**Figure 1d**). Approximately 90% (range 60–93%) of the $CD137^+$ cells were positive for Foxp3 and CD25 and negative for CD127 (**Figure 1d** and **e**). In addition, the majority of the antigen-reactive $CD137^+$ $CD154^-Foxp3^+$ cells co-expressed the transcription factor Helios (**Figure 1f**). FACS-sorted $CD137^+CD154^-$ cells had a highly demethylated Treg-specific demethylated region (TSDR), at levels comparable to $CD25^+CD127^{dim}$-sorted $CD4^+$ T cells or Staphylococcal enterotoxin B (SEB)-stimulated $CD137^+CD154^-$ $CD4^+$ cells. In contrast, $CD154^+$ $CD137^-$ T cells had a methylated Foxp3 locus (**Figure 1g**) and a phenotype matching that of Tcon ($Foxp3^-CD25^-$ $CD127^+$).

Analysis of CD45RO and CCR7 expressions on the fungus-specific $CD137^+CD154^-$ Treg revealed a memory phenotype with a higher frequency of cells in the $CCR7^-$ effector memory compartment (**Figure 2a** and **b**). Further analysis of cytokine expression demonstrated that production of the cytokines IL-2, IL-10, IL-17, TNF-α and IFN-γ is restricted to the fungus-specific $CD154^+$ Tcon, but lacking in $CD137^+$ Treg (**Figure 2c** and **Supplementary Figure S1** online).

In summary, these data confirm that the combined analysis of $CD137^+$ and $CD154^+$ expression can identify fungus-specific memory Treg as well as Tcon in human peripheral blood. The direct comparison between both fungi further suggests that *A. fumigatus* has a stronger potential to induce and/or expand Treg, whereas *C. albicans* induces a stronger Tcon response.

Fungus-reactive $CD137^+$ Treg can be expanded and maintain strong antigen-specific suppressive potential

To proof specificity and function of the fungus-specific Treg, we expanded magnetically enriched $CD137^+$ T cells in the presence of IL-2 and rapamycin for up to 3 weeks. To determine specific reactivity, CD137 expression was analyzed upon re-challenge with the specific or control antigens in the presence of autologous APCs. As shown in **Figure 3a**, fungus-specific Treg specifically upregulated $CD137^+$ expression after restimulation with the specific fungal lysate but not in response to irrelevant antigens. In contrast, polyclonally expanded $CD25^+$ $CD127^{dim}$ Treg reacted strongly only upon stimulation with the high control SEB. Interestingly, we also saw low reactivity against *A. fumigatus* in the polyclonal expanded Treg cell lines (**Figure 3a**), which is consistent with the high frequency

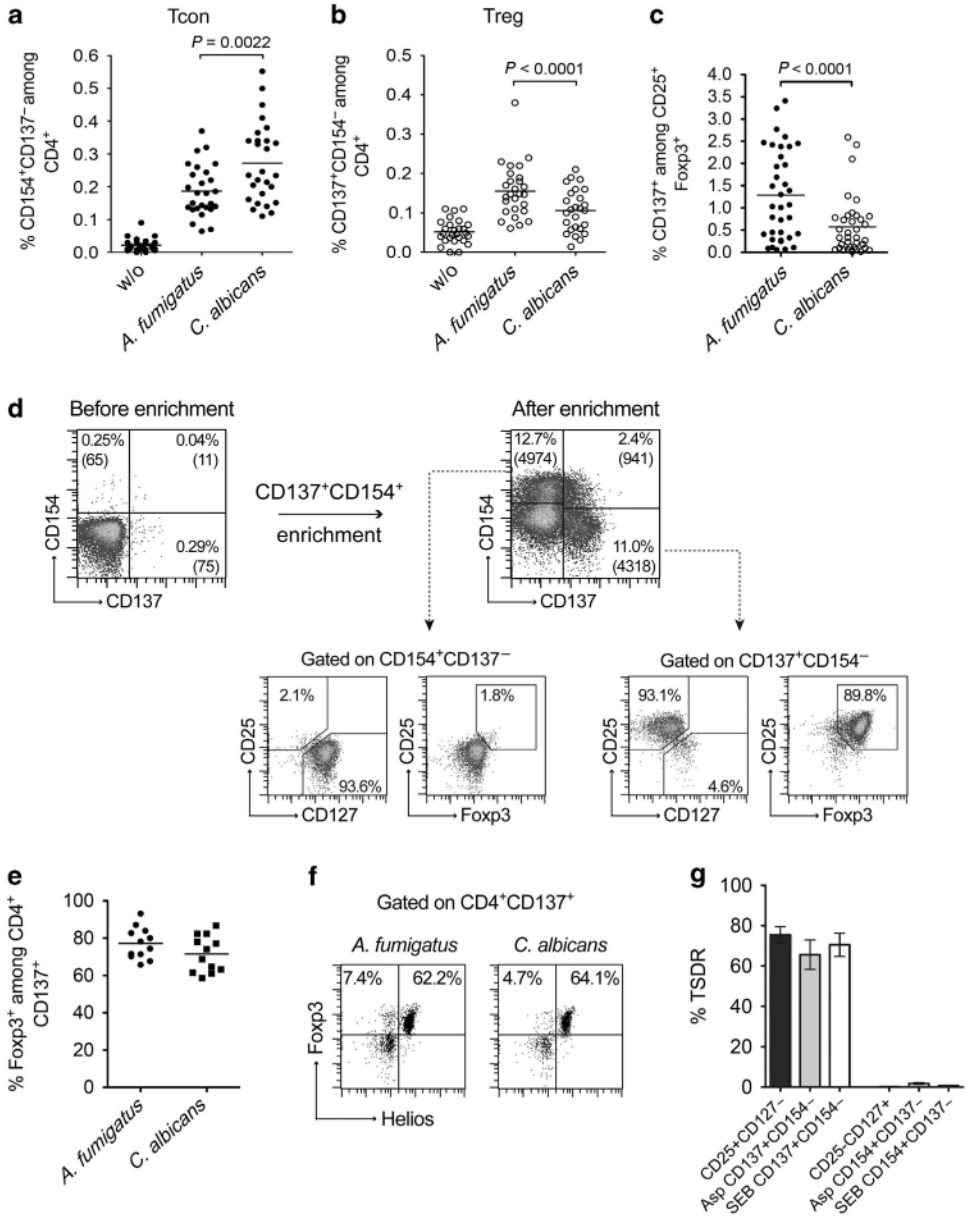

Figure 1 Combined analysis of CD154 and CD137 expression identifies in parallel fungus-specific Tcon and Treg. (**a**, **b**) PBMCs were stimulated with the indicated antigens and analyzed for CD154 and CD137 expression. For an optimized quantification of activation marker positive events among $CD3^+CD4^+$ T cells, cell aggregates (scatter area vs. scatter height), dead cells and non-target cells ($CD14^+$, $CD20^+$, $CD8^+$, dump) were excluded, and cells were counterstained for the activation marker CD69. Summary of several donors is shown with horizontal lines indicating mean values. (**c**) Frequencies of reactive $CD137^+$ cells among $CD25^+Foxp3^+$ $CD4^+$ T cells in PBMC. Panels **a**–**c** show the summary from seven independent experiments with $n=27$ donors. Statistical significance was determined by two-tailed paired Student's *t*-test. (**d**) Expressions of CD154 and CD137 on $CD4^+$ T cells following short-term stimulation of PBMC with *A. fumigatus* lysate before enrichment and after combined $CD154^+/CD137^+$ enrichment. Analysis of CD25, CD127 and Foxp3 expression among enriched $CD154^+CD137^-CD4^+$ Tcon vs. $CD137^+CD154^-CD4^+$ Treg was performed. (**e**) Percentage of Foxp3 expression among enriched $CD137^+CD4^+$ cells in several donors, following short-term stimulation with the fungal lysates. Data from three independent experiments are shown ($n=12$). (**f**) Representative dot plot examples for Helios expression in enriched $CD137^+CD4^+$ cells. Percentages among $CD137^+CD4^+$ are indicated. Six independent experiments with several donors were performed. (**g**) Polyclonal Treg and Tcon as well as *A. fumigatus* or SEB stimulated $CD137^+CD154^-$ or $CD154^+CD137^-$ $CD4^+$ T cells were purified by FACS and analyzed for percentage of demethylation of the Foxp3 Treg-specific demethylated region (TSDR). Graph represents mean ± s.e.m. from five different donors. Two independent sorting experiments were performed.

of *A. fumigatus*-specific Treg in peripheral blood reported here.

We further analyzed whether the expanded Treg exert antigen-specific suppressive function *in vitro*. When *A. fumigatus* antigen was added to the co-culture of allogeneic $CD4^+$ T cells with autologous APCs, the *A. fumigatus*-specific Treg suppressed allo-specific $CD4^+$ T-cell proliferation already at a 1:16 ratio, whereas polyclonal Treg exerted similar

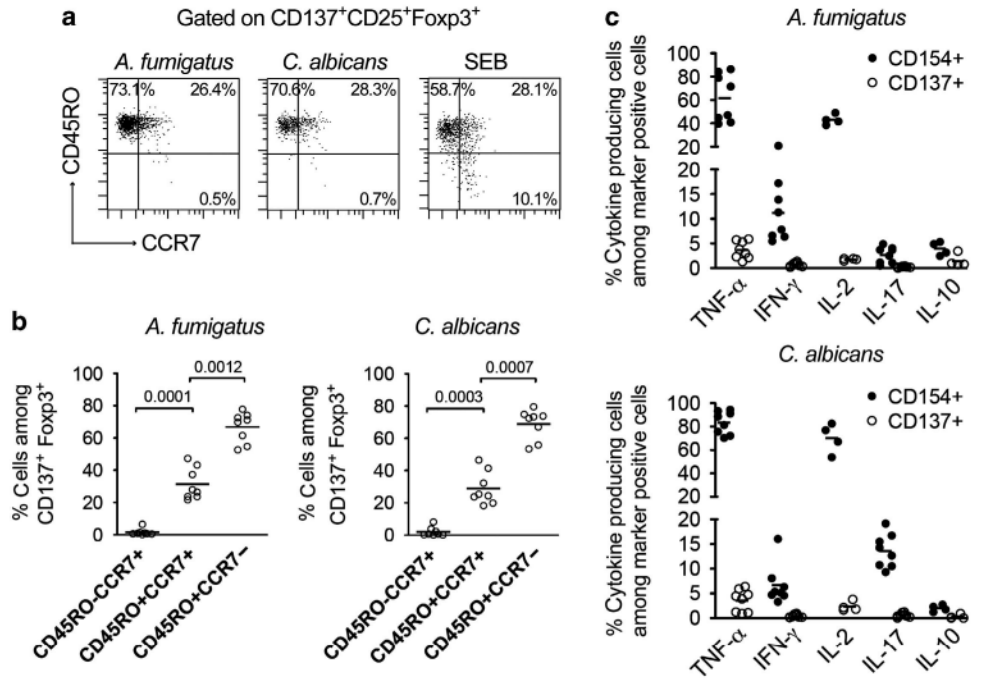

Figure 2 Fungus-specific CD137$^+$ Treg have a memory phenotype and do not produce effector cytokines. Flow cytometric *ex vivo* characterization of magnetically enriched CD154$^+$ or CD137$^+$ T cells from short-term stimulated PBMCs. (**a**, **b**) Phenotypic characterization of fungus stimulated Treg or SEB as a high control. Enriched CD137$^+$CD4$^+$ T cells were gated on CD25$^+$Foxp3$^+$ and analyzed for the expression of CD45RO and CCR7. (**a**) Representative dot plot examples and (**b**) statistical analysis for several donors ($n=8$, two independent experiments were performed). Statistical significance was determined by two-tailed paired Student's *t*-test. (**c**) Fungus-specific T cells were analyzed for cytokine expression. Percentages of cytokine-expressing cells among activation marker positive CD4$^+$ T cells are shown for several donors ($n=8$, two independent experiments were performed; for IL-2: one experiment with four different donors was performed).

suppressive activity only at a 1:1 ratio (**Figure 3b** and **Supplementary Figure 2**). As known for *in vitro* suppression assays, the suppressive activity of both populations was dependent on stimulation with antigen. The fact that also the weak suppressive activity of the polyclonal Treg populations is antigen-dependent again shows that polyclonal Treg contain a relatively high frequency of *A. fumigatus*-specific Treg. In contrast, both Treg populations exerted similar suppressive activity after activation with CD3/CD28 beads. As a control, expanded *A. fumigatus*-specific CD154$^+$ Tcon did not show comparable suppressive capacity following CD3/CD28 stimulation and only modestly inhibited when stimulated with *A. fumigatus* antigens, probably due to the massive expansion of the antigen-specific CD154 cells and competition for nutrients or growth factors as reported before.[20] We also analyzed suppression of CD154 and TNF-α expressions following short-term stimulation of antigen-specific T cells, which is less sensitive to competitive effects due to the short stimulation time. Here *A. fumigatus*-specific Treg as well as polyclonal Treg, but not CD154 Tcon, showed suppressive activity (**Supplementary Figure 3**). Analysis of Foxp3 and Helios expressions during 3 weeks of *in vitro* expansion revealed that, despite the high suppressive activity, the *A. fumigatus*-specific Treg lost Foxp3 expression by about 50%, accompanied by a loss of the demethylated TSDR region and Helios expression, in contrast to polyclonally expanded Treg (**Figure 3c** and **d**). Comparable suppressive capacity and loss of Foxp3 and Helios expressions were observed for the expanded *C. albicans*-reactive Treg (**Supplementary Figure 4**).

These data demonstrate the specificity as well as the strong antigen-specific suppressive capacity of the isolated fungus-specific CD137$^+$ Treg. However, the reduction in Foxp3 expression and the loss of Helios and TSDR demethylation during long-term *in vitro* expansion might indicate that at least a subset of the specific Treg has limited stability, although we cannot exclude selective outgrowth of contaminating Tcon as an alternative explanation.

Minimal peripheral expansion of *A. fumigatus*-specific Tcon and preservation of the naive repertoire

Our data revealed so far a stronger potential of *A. fumigatus* to induce or expand antigen-specific Treg, whereas the potential to induce antigen-specific Tcon was diminished compared with *C. albicans*. To further explore potential differences between *A. fumigatus* and *C. albicans*-specific Tcon, we analyzed the expression of the surface markers CD45RO and CCR7 on CD154$^+$ cells to distinguish between naive and memory T cells. As expected for peripherally expanded T cells, *C. albicans*-reactive Tcon mainly displayed a CD45RO$^+$ memory phenotype (**Figure 4a** and **b**) and were found preferentially within the CCR7$^+$ central memory compartment. For

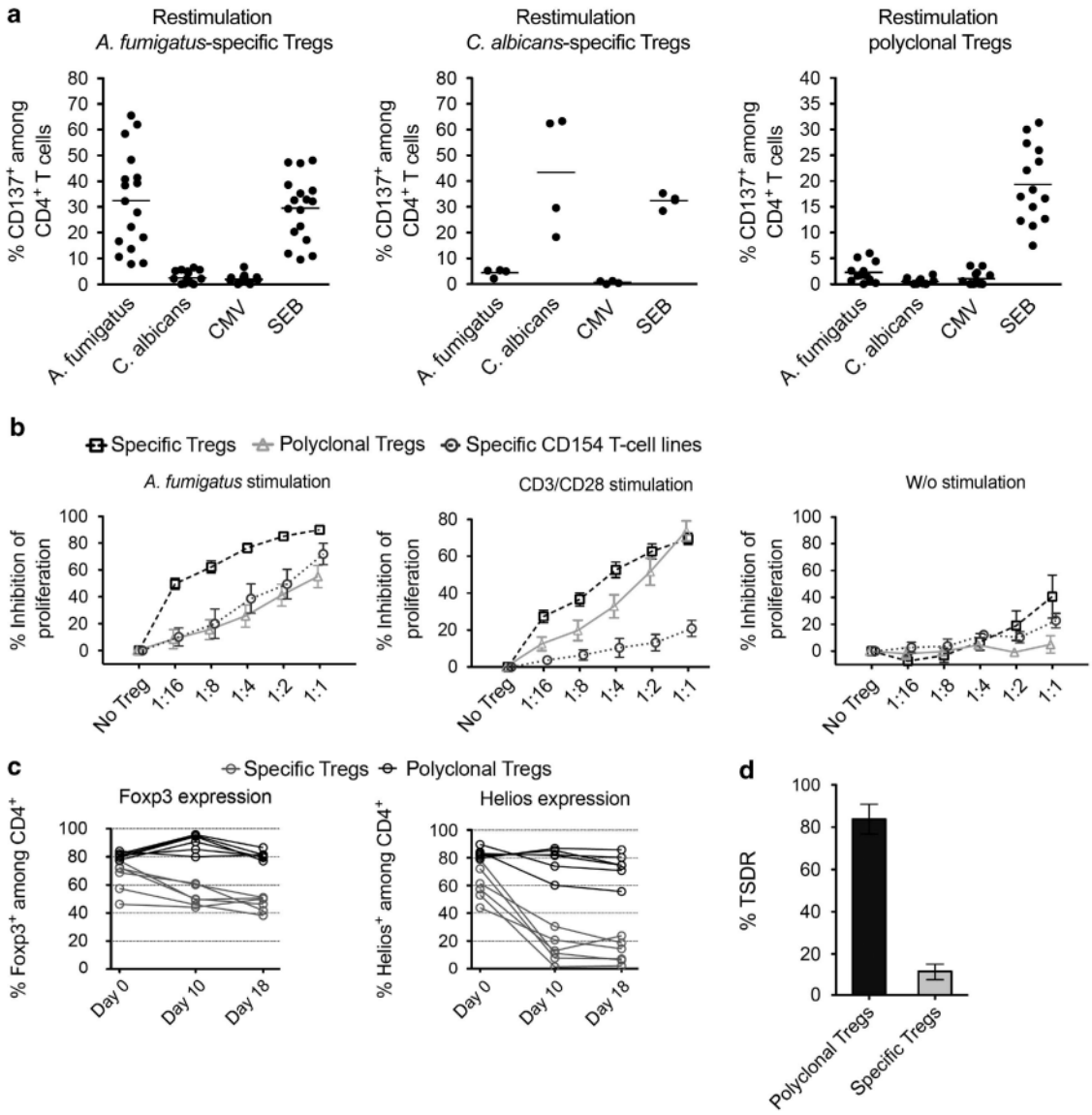

Figure 3 Specificity and suppressive capacity of fungus-specific Treg. Fungal lysate-stimulated CD137$^+$ Treg or polyclonal CD25$^+$CD127^{low} Treg were magnetically isolated and subsequently expanded with IL-2 and rapamycin. (**a**) Expanded Treg were restimulated after 3 weeks of expansion in presence of autologous APCs with and without antigens as indicated and analyzed for re-expression of CD137. Statistical analysis of several donors with percentage of CD137$^+$ cells among CD4$^+$ lymphocytes is shown (*A. fumigatus* Treg: $n=17$, four independent experiments were performed; *C. albicans* Treg: $n=4$, one experiment was performed; polyclonal Treg: n = 14, three independent experiments were performed). (**b**) Expanded *A. fumigatus*-specific CD137$^+$ Treg lines, *A. fumigatus*-specific CD154$^+$ Tcon lines or polyclonal Treg lines were combined with autologous APCs and proliferation dye labeled allogeneic responder CD4$^+$ T cells (Tresp) in different Treg to Tresp ratios. Percentage of inhibition of Tresp proliferation is shown without stimulation or after specific stimulation with *A. fumigatus* lysate or polyclonal stimulation with CD3/CD28. Graph represents mean ± s.e.m. for specific and polyclonal Treg: $n=11$, three independent experiments were performed; specific CD154$^+$ Tcon: $n=6$, two independent experiments were performed. (**c**) Percentage of Foxp3 and Helios expression among CD4$^+$ T cells was analyzed by flow cytometry during expansion of *A. fumigatus*-specific CD137$^+$ Treg or polyclonal CD25$^+$CD127^{dim} Treg. Representative data from two independent experiments are shown, $n=6$. (**d**) Demethylation analysis of the Foxp3 Treg-specific demethylated region (TSDR) in expanded *A. fumigatus*-specific Treg or polyclonal CD25$^+$CD127^{dim} Treg. Graph represents mean ± s.e.m. from 12 different donors. Four independent expansion experiments were performed.

A. fumigatus-specific Tcon, the distribution between the two memory subsets was variable between different donors. Most interestingly, however, 20–70% of all *A. fumigatus*-reactive Tcon displayed a naive CD45RO$^-$CCR7$^+$ phenotype. Further characterization indicated that these cells represent genuine naive T cells as demonstrated by their expression of CD45RA, CD27, a mixed phenotype for CD31,[21] and lack of CD95 and CD11a expression. Furthermore, they did not secrete effector cytokines like IFN-γ or IL-17 (**Supplementary Figure 5**). The specificity of the fungus-specific CD154$^+$ T cells within the naive and memory compartment was confirmed by specific restimulation of expanded CD154$^+$ cells from highly purified naive and memory CD4$^+$ T cells (**Supplementary Figure 6**). This shows that, despite chronic

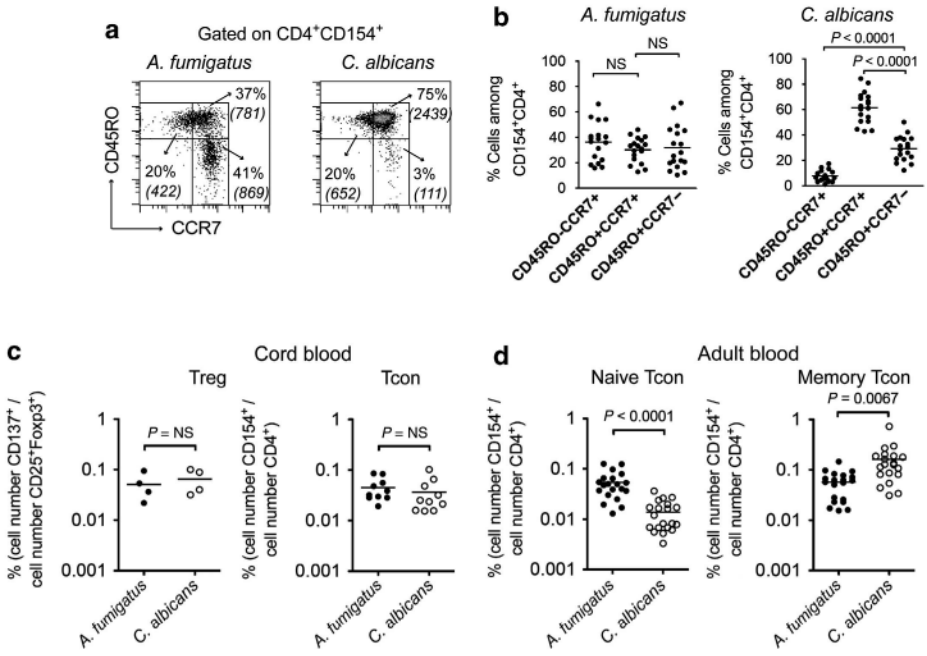

Figure 4 A high proportion of *A. fumigatus*-reactive Tcon is in a naive state. (**a**, **b**) *Ex vivo* analysis of CD45RO and CCR7 expression on magnetically enriched $CD154^+CD4^+$ Tcon. (**a**) Representative dot plot examples of enriched cells from 1×10^7 PBMCs. Percentages of cells among $CD154^+$ and cell counts (in brackets) are indicated. (**b**) Phenotypic characterization of reactive $CD154^+CD4^+$ Tcon in several donors. Percentages of cells among $CD154^+CD4^+$ are indicated. Data from five independent experiments are shown, $n = 18$. (**c**) Enumeration of fungus-reactive Treg and Tcon in cord blood. Frequencies of reactive $CD137^+$ cells among $CD25^+Foxp3^+$ $CD4^+$ T cells ($n = 4$, four independent experiments were performed) and $CD154^+$ cells among $CD4^+$ T cells ($n = 10$, six independent experiments were performed). (**d**) Enumeration of fungus-reactive naive and memory Tcon in adult blood. Frequencies of reactive $CD154^+CD45RO^-CCR7^+$ (naive) and $CD154^+CD45RO^+$ (memory) cells among $CD4^+$ T cells are indicated. Data from five independent experiments are shown, $n = 20$. Frequencies as in panels **c** and **d** were calculated from the total number of $CD154^+CD4^+$ or $CD137^+CD4^+$ cells obtained after enrichment normalized to the total number of $CD4^+$ cells or $CD25^+Foxp3^+CD4^+$ cells applied on the column. Background enriched from the non-stimulated control was subtracted. Statistical significance was determined by two-tailed paired Student's *t*-test.

exposure to *A. fumigatus*, large parts of the Tcon repertoire are left untouched in the naive state.

To corroborate that the enlarged memory Treg and Tcon populations in adult blood are indeed expanded by antigen contact in the periphery, we next analyzed their frequencies in cord blood. As shown in **Figure 4c**, the frequencies of fungus-specific Treg in cord blood were well below 0.1%, corresponding to only 0.002–0.01% within the $CD4^+$ T-cell population (data not shown). This demonstrates indeed a strong peripheral expansion or induction of fungus-specific Treg during adult life. Interestingly, the Tcon as well as Treg frequencies in cord blood were comparable for both fungi, indicating similar amounts of specific precursors in both repertoires (**Figure 4c**). However, the Tcon frequencies against *A. fumigatus* in cord blood were almost identical to those in adult blood (**Figure 4d**). This observation is consistent with the large fraction of naive Tcon in adult blood, and in summary indicates minimal peripheral expansion of *A. fumigatus*-specific Tcon. In contrast, the *C. albicans*-specific Tcon displayed all features of classical memory formation as the memory pool was expanded 5- to 10-fold in adult vs. cord blood, whereas the naive Tcon were reduced by a similar factor (**Figure 4d**).

Taken together, these data confirm that both fungi, but in particular *A. fumigatus*, have the capacity to strongly induce or expand specific memory Treg in the periphery. However, whereas *C. albicans* also induces a Tmem response, the establishment of a Tmem against *A. fumigatus* seems to be limited, pointing toward a unique potential of *A. fumigatus* to circumvent and/or control Tmem responses via selective induction of Treg.

A. fumigatus-specific Treg outnumber and functionally suppress Tmem

To predict the actual impact of expanded Treg on the fungus-specific T-cell response, we determined the absolute numbers of fungus-specific Treg vs. naive and memory Tcon in adult blood. Although the total Treg compartment represents only 5–10% of the $CD4^+$ T cells, the absolute numbers of *A. fumigatus*-specific Treg were equal and for some donors even exceeded the absolute number of naive or memory Tcon (**Figure 5a**). The dominance of the *A. fumigatus* Treg is further exemplified by calculating the ratio of the absolute numbers of Treg vs. Tmem for each individual donor. As shown in **Figure 5b**, the *A. fumigatus*-specific Treg indeed exceeded Tmem by a mean factor of 1.5 (range 0.15–4.2). In sharp contrast, the *C. albicans*-specific T-cell pool was clearly dominated by Tmem, resulting in a reversed Treg/Tmem ratio (mean 0.38, range 0.03–1). Thus, *A. fumigatus*-specific Treg are strongly expanded in the periphery and can even represent the dominant population within the overall repertoire of *A. fumigatus*-specific memory

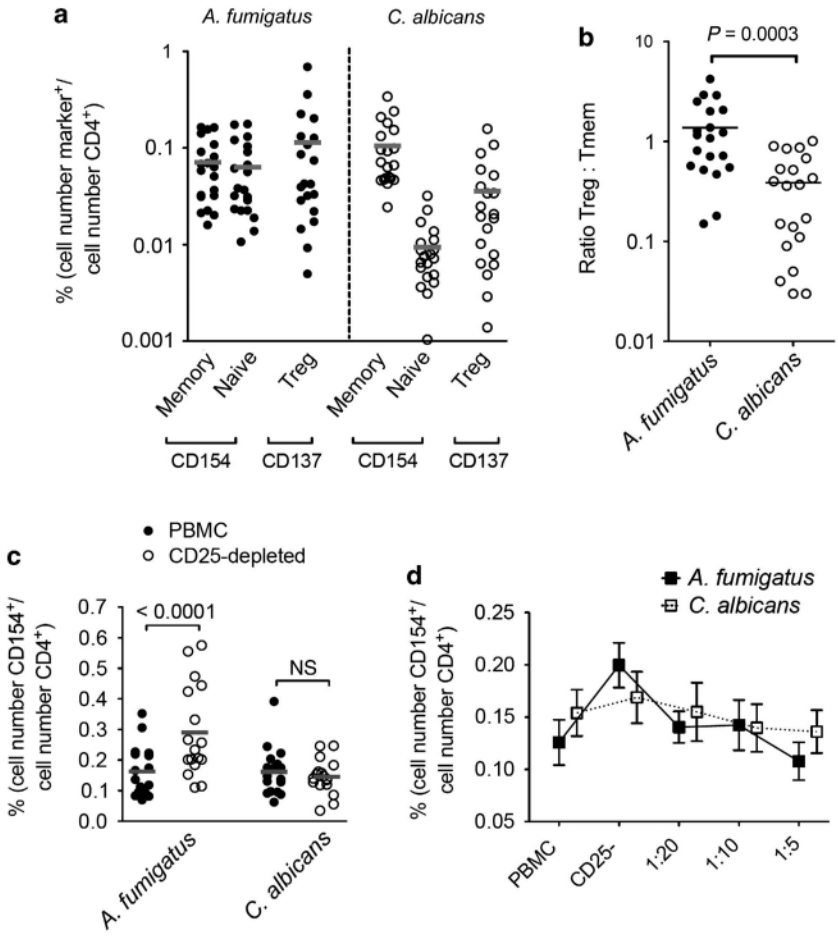

Figure 5 Reversed Treg/Tmem ratios of *A. fumigatus*- and *C. albicans*-specific T-cell responses. (**a**) A total of 1×10^7 PBMCs was stimulated with *A. fumigatus* or *C. albicans* and the numbers of $CD154^+CD137^-$ naive and memory Tcon or $CD137^+CD154^-$ Treg following combined $CD154^+/CD137^+$ enrichment were analyzed. Frequencies were calculated from the total number of $CD154^+$ or $CD137^+$ cells obtained after enrichment, normalized to the total number of $CD4^+$ cells applied on the column. Background enriched from the non-stimulated control was subtracted. (**b**) The ratio of antigen-specific $CD137^+CD154^-$ Treg to $CD154^+CD45RO^+$ Tmem cells is shown. Panels **a** and **b** show data from five independent experiments, $n = 20$. (**c**) Frequencies of antigen-reactive $CD154^+$ Tcon were determined as described in panel **a**, in total PBMCs or after pre-depletion of $CD25^{high}$ cells ($n = 17$, five independent experiments were performed). (**d**) $CD25^{high}$ cells were added back to CD25 pre-depleted PBMCs with the indicated Treg to Tcon ratios. Data show mean ± s.e.m. for six donors; two independent experiments were performed. Statistical significance was determined by two-tailed paired Student's *t*-test.

$CD4^+$ T cells, whereas *C. albicans* induces mainly Tmem responses with minor contribution of Treg.

To confirm these data on a functional level, we analyzed the contribution of Treg to the anti-fungal T-cell response by depleting $CD25^{high}$ cells from PBMCs before antigen stimulation. In line with the idea that Treg control mainly the response against *A. fumigatus*, we found a significant increase in *A. fumigatus*-specific Tcon frequencies, whereas the frequency of *C. albicans*-reactive cells was not significantly altered (**Figure 5c**). In addition, when $CD25^{high}CD4^+$ T cells were added back into the CD25-depleted fraction, the frequency of *A. fumigatus*-specific Tcon dropped to the original level (**Figure 5d**).

Taken together, these results demonstrate that *A. fumigatus* triggers the selective expansion of an antigen-specific Treg pool in healthy human donors, which suppresses Tcon activation *in vitro*, and thus may contribute to tolerance by limiting the formation of an expanded Tmem pool.

Cystic fibrosis patients with *Aspergillus*-associated allergies are characterized by a shift of the *A. fumigatus*-specific Treg/Tmem ratio

To identify a potential role of *A. fumigatus*-specific Treg in mediation of tolerance *in vivo*, we determined whether this protective Treg/Tmem ratio is altered under pathological conditions. To this end we analyzed samples from patients with CF (**Supplementary Table 1** online), which are known to frequently suffer from lung infections and severe allergic reactions against *A. fumigatus*. We were able to identify CF patients with severe allergic reactions against *A. fumigatus* based on a strong Th2 effector cytokine expression of *A. fumigatus*-reactive $CD154^+$ Tcon (i.e. production of IL-13, IL-4 and IL-5) (**Figure 6a** and **b**), which also corresponded with *Aspergillus*-specific IgE levels (**Figure 6c**). By contrast, in non-Th2 CF patients the cytokine profiles of $CD154^+$ *A. fumigatus*-specific Tcon did not differ from those of healthy donors, i.e., they produced mainly TNF-α and IFN-γ but no Th2 cytokines. Strikingly,

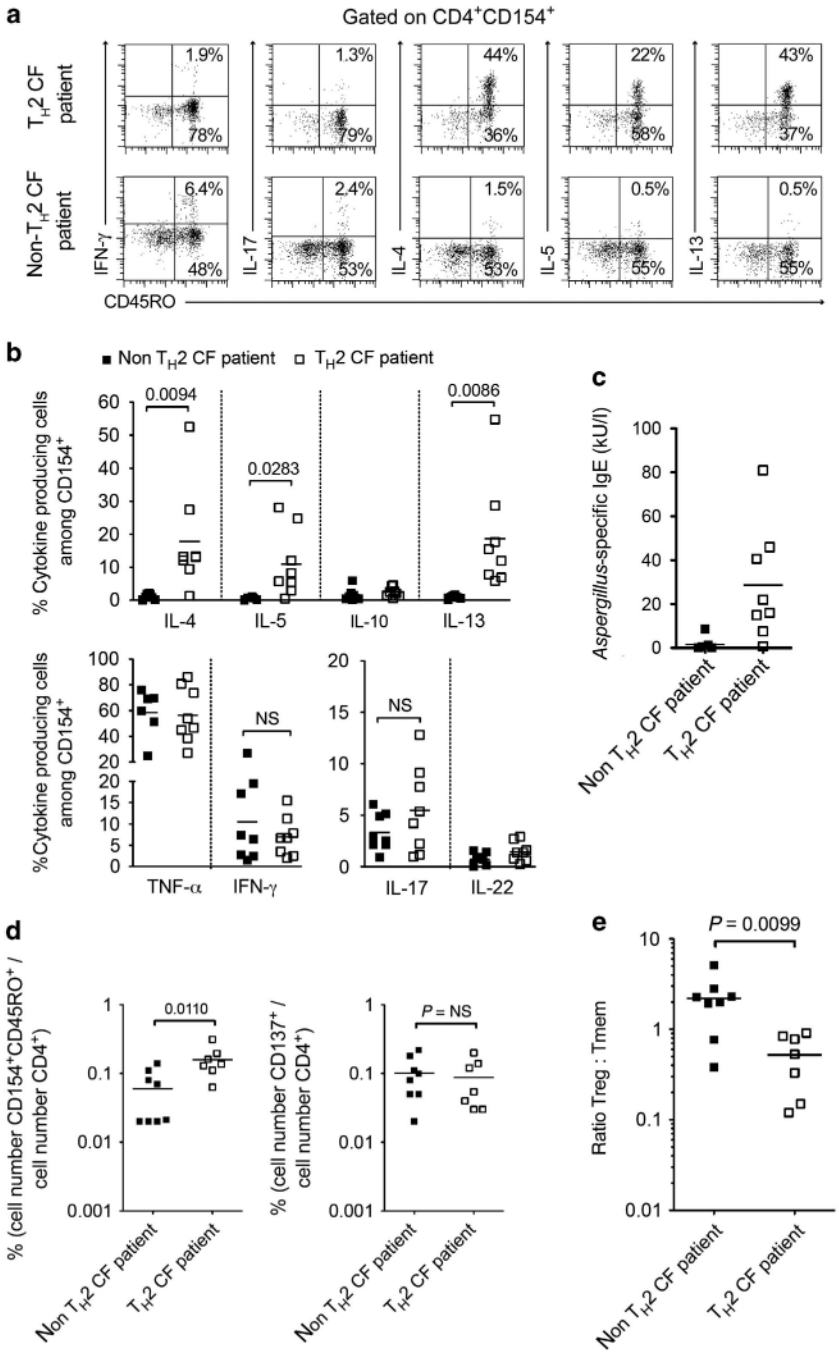

Figure 6 Frequencies of *A. fumigatus*-specific Tmem and Treg in peripheral blood of cystic fibrosis patients. (**a**, **b**) PBMCs from CF patients were stimulated with *A. fumigatus* lysate. Reactive CD154$^+$ Tcon were magnetically enriched and *ex vivo* analyzed for cytokine expression. CF patients were classified according to *A. fumigatus*-specific cytokine expression in Th2 and non-Th2 patients using a cutoff value of $>5\%$ IL-13 producing *A. fumigatus*-reactive CD154$^+$ T cells. (**a**) Representative dot plot examples and (**b**) statistical analysis with percentages of cytokine-expressing cells among CD154$^+$CD4$^+$ are shown. (**c**) *A. fumigatus*-specific serum IgE levels. (**d**) Frequencies of CD154$^+$CD45RO$^+$ Tmem among CD4$^+$ T cells and *A. fumigatus*-reactive CD137$^+$CD154$^-$ Treg among CD4$^+$ T cells. (**e**) Ratio of *A. fumigatus*-specific Treg to Tmem. Panels **b**–**e** show a summary from eight non-Th2 CF patients and eight Th2 CF patients; 13 independent experiments were performed. Statistical significance was determined using two-tailed unpaired Student's *t*-test.

comparing the frequencies of Treg and Tmem cells in both patient groups yielded a two- to fourfold increase of *A. fumigatus*-specific Tmem in Th2 CF patients vs. non-Th2 patients and healthy controls (**Figure 6d** and data not shown). However, fungus-specific Treg frequencies were similar in both patients groups (**Figure 6d**). Thus, the overall ratio of *A. fumigatus*-specific Treg to Tmem was completely reversed in the Th2 CF patient group toward a dominance of Tmem (mean Treg/Tmem 0.52, range 0.12–0.91) (**Figure 6e**). These data highlight that the physiological dominance of specific Treg vs. Tmem in healthy

subjects probably represents an important mechanism to prevent allergy induction.

DISCUSSION

Humans are continuously challenged by various pathogenic fungi, which are either part of the commensal microbiota like *C. albicans* or present in the environment, e.g., in the air that we breath, like *A. fumigatus*. This requires balanced pathogen-specific control mechanisms, which allow effective protection but prevent collateral tissue damage caused by chronic inflammation or allergy. How the human immune system achieves this balance for the various fungal species is currently not well understood. We addressed this question by applying a parallel quantitative assessment of human fungus-specific $CD4^+$ T cells within the naive, memory and Treg repertoire. This approach revealed that both fungi generate unexpectedly high frequencies of specific Treg. Surprisingly, for *A. fumigatus* this was not accompanied by a parallel increase in Tmem, and thus resulted in a Treg dominance characterized by a high Treg/Tmem ratio (mean 1.5), as well as preservation of a large naive Tcon population and significant suppression of *A. fumigatus*-specific T-cell activation *in vitro*. The protective Treg/Tmem balance was completely reversed in CF patients with allergic reactions against *A. fumigatus* due to the formation of a strong Th2-type Tmem response. To the best of our knowledge, this is the first direct evidence for the formation of a preferential antigen-specific Treg vs. Tmem response in humans, representing a potential mechanism for the protection from Th2-associated immunopathology against an opportunistic environmental pathogen.

In the majority of donors analyzed, *A. fumigatus*-specific Treg even outnumber specific Tmem. In addition, the specific naive T-cell pool is largely preserved in size when compared with cord blood, suggesting a limited Tcon response against *A. fumigatus* antigens. In contrast, the *C. albicans*-reactive T-cell repertoire mainly consisted of Tmem, which exceeded *A. fumigatus*-specific Tmem by a factor of ~2–5. At the same time, a depletion from the naive pool was observed as it has been reported for other pathogen-specific T-cell responses.[22] Although we clearly detected specific Treg against *C. albicans*, they occurred at lower frequencies than specific Tmem (mean ratio Treg/Tmem 0.38) and *A. fumigatus*-specific Treg. In this respect, the T-cell response against *C. albicans* is similar to several other anti-pathogen T-cell responses, including viruses, parasites and bacteria, where antigen-specific Treg expansion occurs along with a strong effector T-cell response.[23–25] The dominant role of the enlarged *A. fumigatus*- but not *C. albicans*-specific Treg pool is further supported by functional data showing that total Treg depletion results in an increased stimulation of *A. fumigatus* but not *C. albicans*-specific Tcon.

The data reported here clearly demonstrate a fundamental difference between the T-cell responses against *A. fumigatus* and *C. albicans* beyond the previously described differences with regard to effector cytokine production by Tmem.[18,26–29] Whereas *C. albicans* generates a classical Th17-dominated anti-pathogen T-cell response with only a minor contribution of specific Treg, *A. fumigatus* seems to preferentially expand Treg and rather preserves the naive T-cell repertoire. This difference may be due to the nature of the pathogen, e.g., expression of pathogen-associated molecular patterns, the infection route or the antigen dose. As *A. fumigatus* is a ubiquitously distributed airborne fungus, humans acquire it primarily by inhaling spores. In contrast, the commensal *C. albicans* is mainly residing on the mucosal surface of the gastrointestinal tract and skin. However, both the gut and the lung are known as preferential sites for the generation of Treg responses against commensals and environmental antigens.[2,5,6,30–33] As for now, there is no evidence that *A. fumigatus* has an inherent tolerogenic potential but rather has been reported to generate effector T-cell responses after repeated nasal exposure with *A. fumigatus* conidia *in vivo*.[34] Therefore, it seems likely that antigen dosing and timing are relevant parameters for the preferential Treg activation by *A. fumigatus* antigens.

C. albicans invasion usually occurs in a spatially and timely restricted manner, e.g., after epithelial damage,[35] which probably results in a transiently high local antigen concentration and presumably leads to a strong immune reaction. In contrast, the continuous inhalation of airborne *A. fumigatus* spores may rather favor the disseminated and chronic presence of antigens at low concentrations. Furthermore, innate immune recognition of dormant *A. fumigatus* spores is largely prevented by a hydrophobin surface layer,[36,37] which may further diminish antigen uptake and presentation. The chronic exposure to low doses of antigens has previously been described to support the generation of Treg responses.[38–40] In addition, the capacity of lung macrophages to induce Treg from naive T cells has recently been reported for the murine system.[41] This further supports the observed preferential Treg response against inhaled *A. fumigatus* antigens.

Because of the lack of distinctive markers in humans, the origin of the fungus-specific Treg remains speculative. The analysis of cord blood samples clearly showed that *A. fumigatus*-specific, as well as *C. albicans*-specific, Treg and Tcon are present in similar but very low frequencies at birth, proving that the enlarged Treg pool is the result of an antigen-specific induction or expansion process during life. This is also in accordance with the $CD45RO^+$ memory phenotype of the fungus-specific Treg. However, the question whether the specific Treg populations arise from expanded thymic (tTreg) or peripherally induced Treg (pTreg) remains. Our *ex vivo* phenotypic analysis revealed hallmarks of tTreg, i.e., fully demethylated TSDR, expression of Helios, lack of cytokine and CD127 expression. A thymic origin would also be in accordance with our finding that in adult blood the naive Tcon pool was not significantly depleted of *A. fumigatus*-reactive cells. Moreover, tTreg have recently been shown to significantly contribute to the T-cell repertoire specific for intestinal commensal microbiota.[2] In contrast, pTreg formation against airborne antigens seems to be essential for the maintenance of tolerance at mucosal sites, as mice specifically lacking pTreg selectively develop pathological Th2 responses at

mucosal surfaces.[5] This leaves open the possibility that the fungus-specific Treg population is actually composed of pTreg as well as expanded tTreg. This could also explain why Helios and Foxp3 expressions, as well as TSDR demethylation, were lost at least by a subset of fungus-reactive Treg upon prolonged *in vitro* expansion. Such phenotype instability has been described for *in vitro*-induced Treg[42] and long-term cultured $CD25^+CD127^-CD45RO^+$ memory Treg.[43] However, selective outgrowth of effector T cells even in the presence of rapamycin cannot be excluded.

Our study has also important clinical implications, as *A. fumigatus* is the cause of several diseases. In immunocompetent individuals repeated exposure to *A. fumigatus* can trigger pathological Th2 responses associated with strong allergic reactions such as asthma or ABPA. Especially, CF patients frequently suffer from *Aspergillus* colonization of the lung and associated allergic responses.[44] However, the reasons why some patients develop a severe pathologic Th2 response while others do not are currently unknown. Intriguingly, we observed that those CF patients with a strong Th2 bias of the *A. fumigatus*-specific Tcon, which also corresponded with increased *A. fumigatus*-specific IgE levels, displayed a marked shift of the Treg/Tmem ratio (from 2.1 to 0.5). This shift was due to the selective expansion of *A. fumigatus* Th2 Tmem, whereas Treg numbers remained constant, suggesting no direct defect in Treg generation in CF patients. There is also no other evidence that Treg function may generally be affected by the *CFTR* gene mutations underlying cystic fibrosis. Thus, our data suggest that *A. fumigatus*-specific Th2 differentiation and expansion can be initiated by escaping Treg control under the specific conditions of CF lung environment. This escape is most likely related to the increased *A. fumigatus* load[44] due to defective epithelia or phagocyte function,[45] which may lead to alterations in antigen density and APC composition or activation status. In any case, these data provide direct evidence for the hypothesis that Treg formation is an important process to protect from allergy development against chronically inhaled *A. fumigatus* antigens in healthy subjects and that alterations in this process or an imbalance between pathologic Tmem vs. Treg contribute to disease development or chronification, as demonstrated in the murine system.[5,46–48] In line with this, patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, who are deficient for Treg, have indeed elevated levels of IgE, and recently a case of ABPA in an IPEX patient occurring at an unusually young age was reported.[49] In contrast, immunodeficient patients are prone to contracting life-threatening invasive *Aspergillus* infections with high mortality rates (50–90%).[8] For this patient group, the capacity of *A. fumigatus* to preferentially generate a Treg response may even represent a potential risk factor, as it prevents or impairs protective effector T-cell responses. Indeed, increased levels of the immunosuppressive cytokine IL-10 have been associated with a poor disease outcome in patients with invasive aspergillosis,[50] although there is so far no direct evidence for the presence of *A. fumigatus*-specific Treg in these patients.

Beyond the immediate relevance of our findings for human diseases related to *A. fumigatus* infections, delineating the mechanisms how *A. fumigatus* induces selective Treg vs. Tcon responses in humans will help to design therapeutic strategies aiming at the induction of antigen-specific Treg.

METHODS

Blood donors. Buffy coats or peripheral ethylenediamine tetraacetic acid (EDTA) blood samples from healthy human donors were obtained from the Institute for Transfusion Medicine, University Hospital Dortmund, Germany and from in-house volunteers. Peripheral blood samples from 16 patients with a diagnosis of cystic fibrosis (CF) were obtained from the University Hospital Cologne, Germany. Blood sampling was performed within the scope of the biomaterial repository protocol ISI, and all patients gave informed consent (local ethics identifier 08–160). CF patients were classified according to the *A. fumigatus*-stimulated production of cytokines. A cutoff value of >5% IL-13 producing *A. fumigatus*-reactive $CD154^+$ T cells was determined to discriminate between Th2 and non-Th2 patients.

Cell preparation and stimulation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare Life Sciences, Freiburg, Germany) density gradient centrifugation and resuspended in RPMI-1640 (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with 5% (v/v) human AB-serum (BioWhittaker/Lonza, Walkersville, MD), 2 mM L-glutamine (PAA Laboratories, Pasching, Austria). Cells were stimulated with the following antigens: *A. fumigatus* lysate (40 $\mu g\,ml^{-1}$; generated as described),[18] *C. albicans*-lysate (40 $\mu g\,ml^{-1}$; Greer Laboratories, Lenoir, NC), CMV-lysate (10 $\mu g\,ml^{-1}$, Siemens Healthcare Diagnostics, Marburg, Germany), Staphylococcal enterotoxin B (SEB 1 $\mu g\,ml^{-1}$, Sigma Aldrich, Schnelldorf, Germany) in presence of 1 $\mu g\,ml^{-1}$ CD40, 1 $\mu g\,ml^{-1}$ CD28 pure antibodies (both Miltenyi Biotec).

Flow cytometry. Cells were stained in different combinations with the following monoclonal antibodies according to manufacturers' protocols: CD4-VioBlue, CD4-FITC, CD4-PE-Vio770, CD4-APC-Vio770 (VIT4), CD3-VioBlue (BW264/56), CD8-VioGreen, CD8-PerCP (BW135/80), CD14-VioGreen, CD14-PerCP (TÜK4), CD20-VioGreen, CD20-PerCP (LT20), CD69-FITC (FN50), CD137-PE, CD137-APC (4B4-1), CD154-VioBlue, CD154-FITC, CD154-APC (5C8), CD25-PE, CD25-APC (4E3), CD127-FITC (MB15-18C9), CD45RO-PE-Vio770, CD45RO-APC (UCHL1), TNF-α-FITC, TNF-α-PE (cA2), IFN-γ-APC (45-15), IL-17A-APC, IL-17A-FITC (CZ8-23G1), IL-2-APC (N7.48A), IL-10-APC (JES3-9D7), IL-4-PE (7A3-3), IL-5-APC (JES1-39D10), IL-13-PE (JES10-5A2.2), Foxp3-APC (3G3) (all from Miltenyi Biotec), Foxp3-PerCP-Cy5.5 (PCH101) IL-22-PerCP-Cy5.5 (22URTI) (both from eBioscience, San Diego, CA, USA), Helios-FITC, Helios-PE, (22F6), CCR7-Alexa Fluor 488 (G043H7), IFN-γ-PerCP-Cy5.5 (4S.B3) (all from BioLegend, San Diego, CA, USA), TNF-α-VioBlue (cA2), IFN-γ-VioBlue (45-15), HLA-A2-PE (BB7.2) (all conjugated in-house). Data were acquired on a MACSQuant analyzer. MACSQuantify software was used for analysis (both Miltenyi Biotec).

Enrichment and characterization of antigen-reactive T cells. A total of 1×10^7 PBMCs per ml was stimulated for 7 h with different antigens. For combination of antigen-reactive T-cell enrichment with an intracellular staining, 1 $\mu g\,ml^{-1}$ Brefeldin A (Sigma Aldrich) was added for the last 2 h. Cells were separated using the CD154 MicroBead Kit and/or the CD137 MicroBead Kit (both Miltenyi Biotec) either alone or in combination. In brief, cells were indirectly magnetically labeled with CD154-Biotin and CD137-PE, followed by anti-Biotin and anti-PE MicroBeads and enriched by two sequential MS columns (Miltenyi Biotec). Surface staining was performed on the first column,

followed by fixation and intracellular cytokine staining on the second column (Inside stain Kit; Miltenyi Biotec), as described,[18] or staining for transcription factors using the Foxp3 Staining Buffer Set (Miltenyi Biotec). In some experiments, $CD25^{high}$ cells were depleted before stimulation and enrichment of antigen-reactive $CD154^+$ T cells, using CD25 MicroBeads and LD columns (both Miltenyi Biotec).

***In vitro* T-cell expansion and restimulation.** Stimulation and isolation of antigen-specific T cells was performed as described above. Polyclonal Treg were isolated with the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-cell Isolation Kit (Miltenyi Biotec). A total of 1×10^5 per well polyclonal Treg and 1×10^4per well $CD137^+$ cells were cultured in 96-well round-bottom plates in X-Vivo15 (BioWhittaker/Lonza), supplemented with 5% (v/v) human AB-serum, 500 U ml^{-1} IL-2 (Proleukin; Novartis, Nürnberg, Germany), 30 nM rapamycin (Sigma Aldrich), and 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} amphotericin B (Antibiotic Antimycotic Solution, Sigma Aldrich). For polyclonal Treg, 4:1 (bead-to-cell ratio) Treg Expansion Beads (Miltenyi Biotec) were added. Cells were expanded for 21 days, with polyclonal Treg being restimulated 2:1 (bead-to-cell ratio) at day 10.

Isolated $CD154^+$ cells were cultured for 14 days, at a density of 2.5×10^6 cells per cm^2 with 1:100 mitomycin C (Sigma Aldrich) treated autologous feeder cells in X-Vivo15, supplemented with 5% (v/v) AB-serum, 200 U ml^{-1} IL-2 and 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} amphotericin B. During expansion medium was replenished, and cells were split as needed. For expansion of T-cell lines from the naive or memory repertoire, $CD4^+$ T cells were isolated by negative selection using the naive $CD4^+$ T cell-Isolation Kit or the memory $CD4^+$ T cell Isolation Kit (both Miltenyi Biotec), respectively. A total of 1×10^7 isolated naive or memory $CD4^+$ T cells were stimulated with autologous CD3-depleted PBMCs as APCs in a ratio of 1:1 and fungal lysates. Enrichment and cultivation of reactive $CD154^+$ cells from both $CD4^+$ T-cell subsets was performed as described above.

Before restimulation, expanded cells were rested in RPMI-1640 + 5% human AB-serum for 2 days. A total of 5×10^5 expanded cells were re-stimulated with autologous CD3-depleted PBMCs (CD3 MicroBeads, LD columns; both Miltenyi Biotec) in a ratio of 1:1 in 96-well flat bottom plates in presence of 1 µg ml^{-1} CD28 functional grade pure Ab for 2 h plus additionally 4 h with 1 µg ml^{-1} Brefeldin A (Sigma Aldrich). After fixation and permeabilization, cells were stained intracellularly for CD137 and Foxp3 expression (Foxp3 Staining Buffer Set; Miltenyi Biotec).

Stimulation of cord blood samples. Cord blood samples were obtained from local hospitals after informed consent. $CD14^+$ monocytes were isolated from cord blood mononuclear cells (CBMCs) by positive selection with CD14 MicroBeads (Miltenyi Biotec) and cultivated for 2 days with an antigen in X-Vivo15 (BioWhittaker/Lonza), supplemented with 1000 IU ml^{-1} GM-CSF and 400 IU ml^{-1} IL-4 (both Miltenyi Biotec) to generate fastDC. DCs were maturated for 1 day with 1000 IU ml^{-1} TNF-α, 1000 IU ml^{-1} IL1-β (both Miltenyi Biotec), 1 µg ml^{-1} PGE_2 (Sigma Aldrich) and were used for stimulation of the autologous $CD14^-$ fraction. Enrichment of $CD154^+$ and $CD137^+$ T cells was performed as described above.

For restimulation of expanded $CD154^+$ cells from cord blood samples, $CD34^+$ cells were isolated using the CD34 MicroBead Kit (Miltenyi Biotec) and were cultured for 3 weeks in X-Vivo™15 supplemented with 5% (v/v) AB-serum, 25 ng/ml Flt3-L, 10 ng/ml TPO and 20 ng/ml SCF (all Miltenyi Biotec) at a density of 1×10^5/ml. At day 21, $CD14^+$ cells were isolated with CD14 MicroBeads (Miltenyi Biotec) and used for restimulation of the expanded $CD154^+$ T cells.

Proliferation assay. For proliferation assays, $CD4^+$ responder T cells (Tresp) were untouched isolated with the $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) and labeled with the CellTrace Violet Cell Proliferation Kit (Invitrogen, Molecular Probes, Eugene, OR) at a final concentration of 2.5 µM. A total of 2×10^5 Tresp were co-cultured in different ratios with expanded polyclonal or specific Treg or expanded CD154 T cell lines as a control, and 4×10^5 Treg-autologous CD3-depleted PBMCs as APCs and inducer of an allo-reaction. Treg were either stimulated specifically with 40 µg ml^{-1} *A. fumigatus* lysate or polyclonal with anti-CD3/CD28 beads (Treg Suppression Inspector; Miltenyi Biotec). On day 6, dilution of proliferation dye was analyzed by flow cytometry. Tresp could be discriminated from Treg and APCs by using donors with opposite HLA-A2 expression[19] (**Supplementary Figure 2a**).

Suppression of $CD154^+$ induction. Tresp were generated by expansion of *A. fumigatus*-specific $CD154^+$ T cell lines, as described above and stained with the CellTrace Violet Cell Proliferation dye (Invitrogen, Molecular Probes). A total of 2×10^5 Tresp were co-cultured in different ratios with expanded polyclonal or specific Treg from the same donor and 4×10^5 CD3-depleted PBMCs as APCs. Specific T cells were stimulated with *A. fumigatus* lysate and polyclonal Treg with anti-CD3/CD28 beads in presence of 1 µg ml^{-1} CD28 functional grade pure Ab for 2 h plus additionally 4 h with 1 µg ml^{-1} Brefeldin A (Sigma Aldrich) and stained intracellular for CD154 and TNF-α induction. Tresp and Treg could be discriminated by Violet Dye staining of the responder cells (**Supplementary Figure 3a**).

Methylation analysis of Foxp3 Treg-specific demethylated region (TSDR). After centrifugation, cell pellets were dissolved in 400 µl PBS, and genomic DNA was isolated using the QIAamp^R DNA Blood Mini Kit (Qiagen, Hilden, Germany). Five-hundred nanograms of eluted genomic DNA was used in a subsequent bisulfite treatment (EpiTect^R, Qiagen). A minimum of 60 ng bisulfite-treated genomic DNA was then used in a real-time-PCR to quantify the FOXP3 TSDR. Real-time-PCR was performed in a final reaction volume of 20 µl containing 10 µl FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany), 50 ng µl^{-1} Lamda DNA (New England Biolabs, Frankfurt, Germany), 5 pmol µl^{-1} methylation or non-methylation specific probe, 30 pmol µl^{-1} methylation or non-methylation specific primers and 60 ng bisulfite-treated DNA or respective amount of plasmid standard. The samples were analyzed in triplicates on an ABI 7500 Cycler using the following cycling conditions: 1 cycle of 10 min 95 °C and 45 cycles of 15 s 95 °C followed by 1 min 61 °C. % FOXP3 TSDR content was then calculated by dividing the non-methylated copy-number by the total genomic FOXP3 copy-number. For detailed instruction see also.[51]

Statistics. Statistical analyses were performed with the GraphPad PRISM software 5.0 (GraphPad Software, La Jolla, CA). Significance was determined by two-tailed Student's *t*-test as indicated. *P*-values of <0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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Figure S1

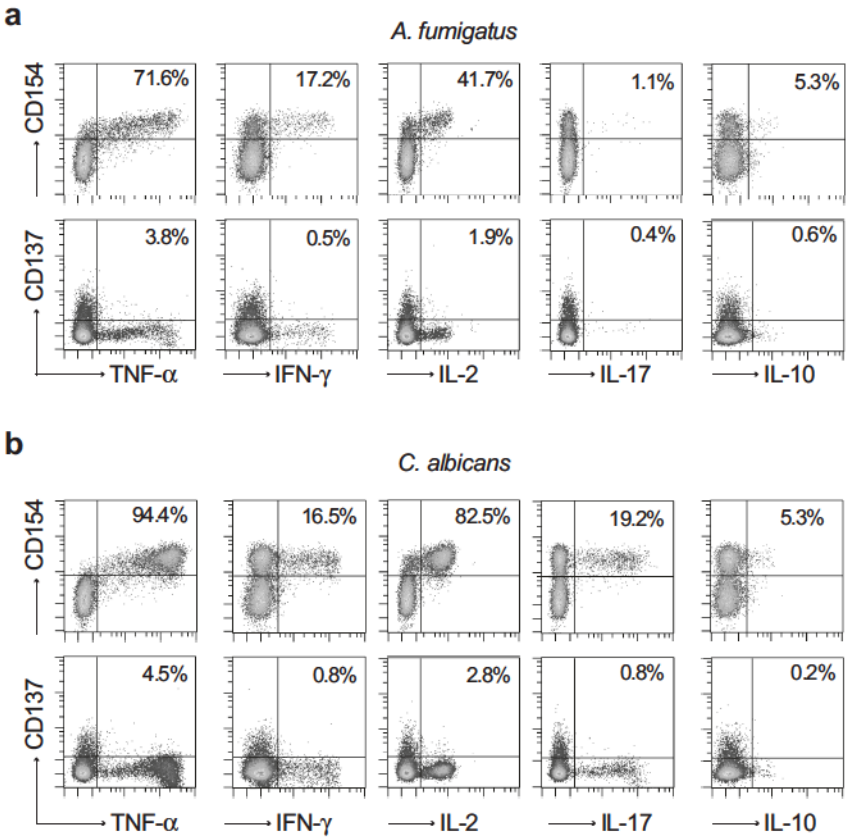

Figure S1. Fungus-specific CD137$^+$ Treg do not produce effector cytokines.

Representative dot plot examples for *ex vivo* cytokine expression of magnetically enriched (**a**) *A. fumigatus*-specific and (**b**) *C. albicans*-specific CD154$^+$ Tcon or CD137$^+$ Treg. Cells are gated on CD4$^+$ lymphocytes and percentages of cytokine-expressing cells among activation marker positive CD4$^+$ T cells are shown.

Figure S2

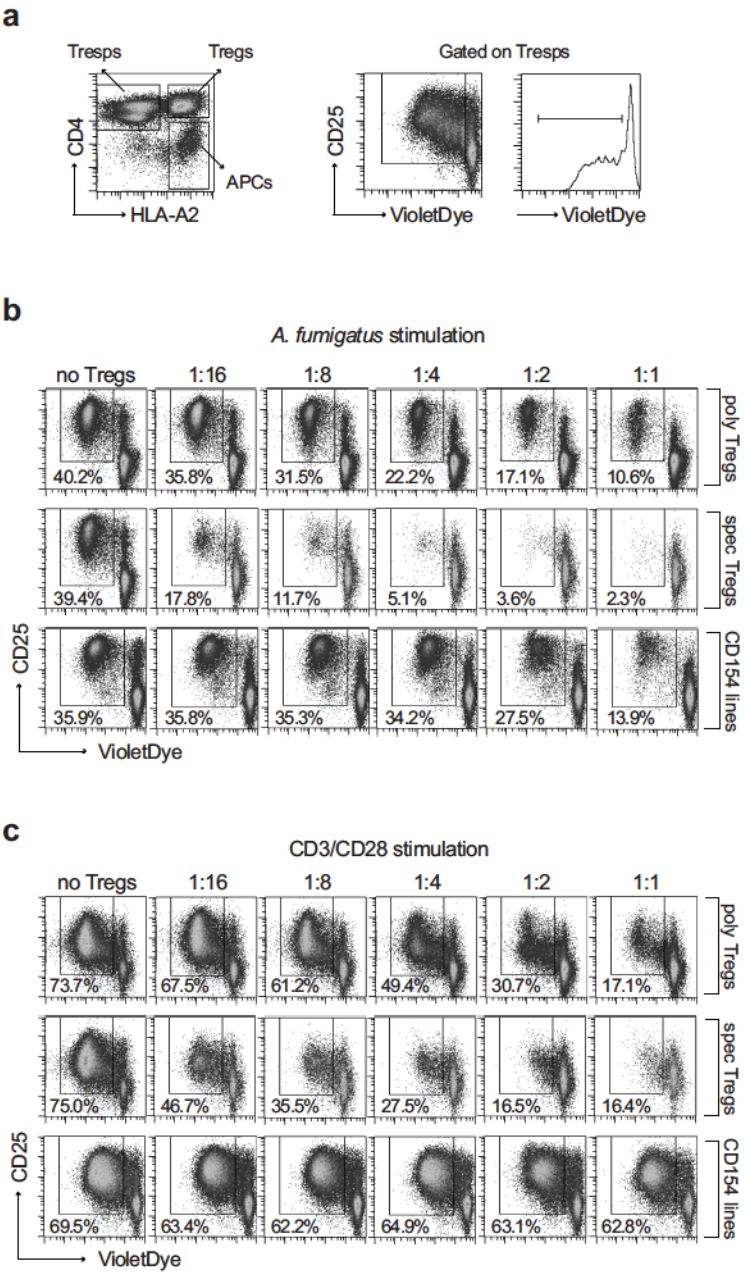

Figure S2. *A. fumigatus*-specific Treg suppress proliferation. (**a**) Experimental set up for proliferation assay using expanded Treg, autologous APCs and proliferation dye labelled allogeneic responder $CD4^+$ T cells (Tresp). Tresp could be discriminated from Treg and APCs by using donors with opposite HLA-A2 expression.[19] (**b, c**) Representative dot plot examples for suppression of allogeneic Tresp proliferation by expanded polyclonal Treg lines, *A. fumigatus*-specific $CD137^+$ Treg lines, or *A. fumigatus*-specific $CD154^+$ Tcon lines after (**b**) specific stimulation with *A. fumigatus* lysate or (**c**) polyclonal stimulation with CD3/CD28. Numbers indicate percentage of proliferating responder cells at different Treg to Tresp ratios.

Figure S3

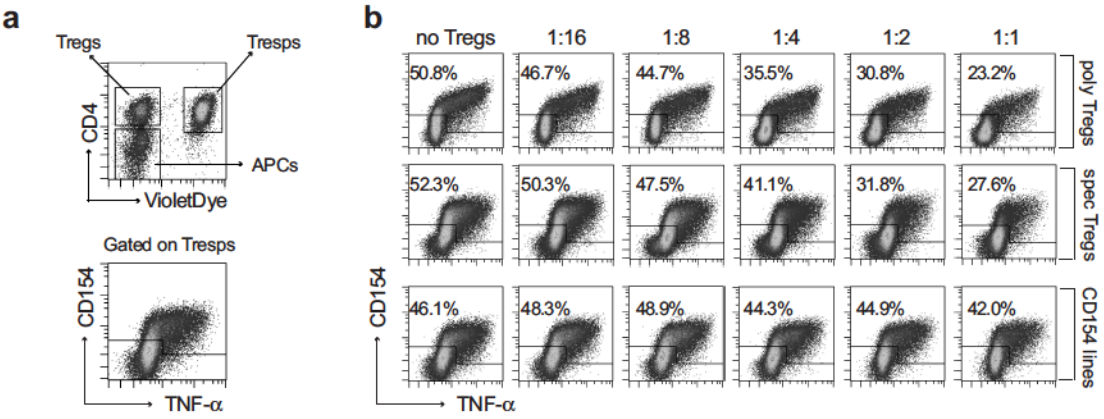

Figure S3. Specific suppression of CD154$^+$ induction.

Expanded *A. fumigatus*-specific CD154$^+$ Tcon lines were used as responder cells (Tresp). Polyclonal or specific CD137$^+$ Treg or specific CD154$^+$ Tcon lines were combined with autologous APCs and Tresp from the same donor in different Treg to Tresp ratios. Following short-term stimulation with *A. fumigatus* lysate for antigen-specific T cell lines or CD3/CD28 for polyclonal Treg, the expression of CD154 and TNF-α was analyzed. (**a**) Experimental set up for the suppression assay. Staining of Tresp with proliferation dye allowed clear discrimination between Treg and Tresp cells. (**b**) Percentage of CD154 and TNF-α expressing cells are indicated.

Figure S4

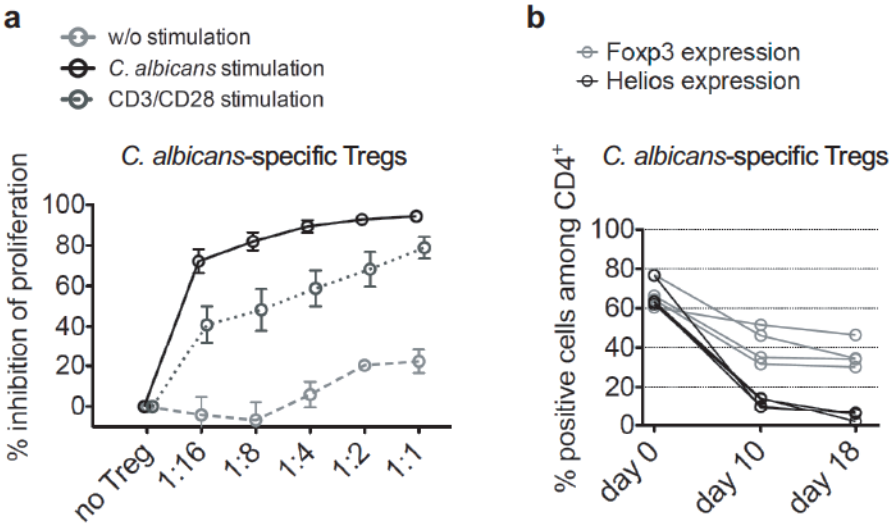

Figure S4. Suppressive capacity of *C. albicans*-specific Treg.

(**a**) Expanded *C. albicans*-specific CD137$^+$ Treg lines were combined with autologous APCs and proliferation dye labelled allogeneic responder CD4$^+$ T cells (Tresp) in different Treg to Tresp ratios. Percentage of inhibition of Tresp proliferation is shown without stimulation or after specific stimulation with *C. albicans* lysate or polyclonal stimulation with CD3/CD28. Graph represents mean ± SEM. (**b**) Percentage of Foxp3 and Helios expression among CD4$^+$ T cells was analyzed by flow cytometry during expansion of *C. albicans*-specific CD137$^+$ Treg. One experiment with four different donors was performed.

Figure S5

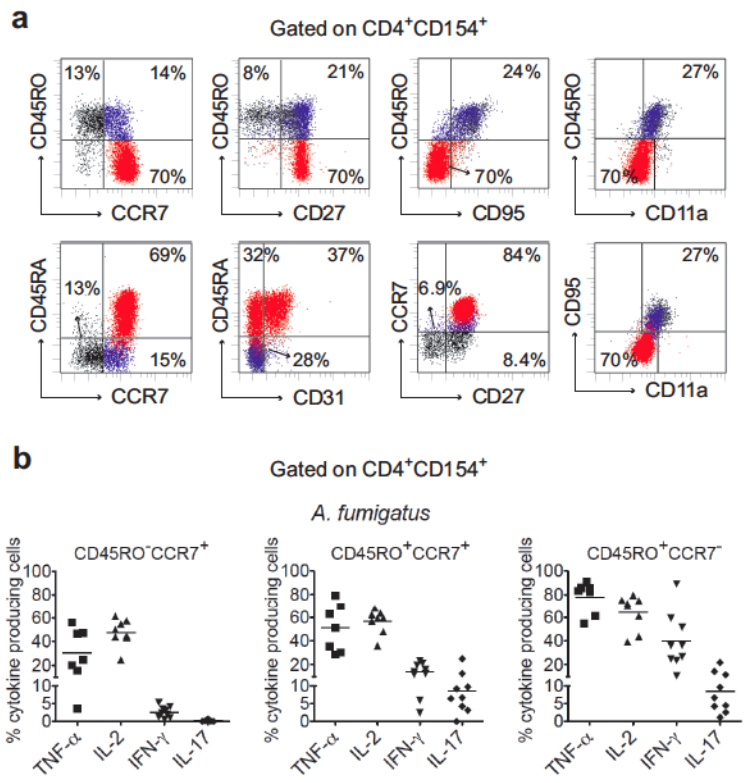

Figure S5. Phenotypic and functional characterization of *A. fumigatus*-specific naive Tcon. (**a**) PBMCs were stimulated with *A. fumigatus* lysate, reactive CD154$^+$ Tcon were magnetically enriched and *ex vivo* analyzed for surface expression of phenotypic markers. Percentages of cells among CD154$^+$CD4$^+$ are indicated. (**b**) *A. fumigatus*-reactive CD154$^+$ Tcon were gated on different subsets according to surface expression of CD45RO and CCR7 and stained intracellularly for cytokine expression. Percentage of cytokine-expressing cells among the indicated phenotypic subsets is shown for several donors, with horizontal lines indicating mean values (n = 9, three independent experiments were performed).

Figure S6

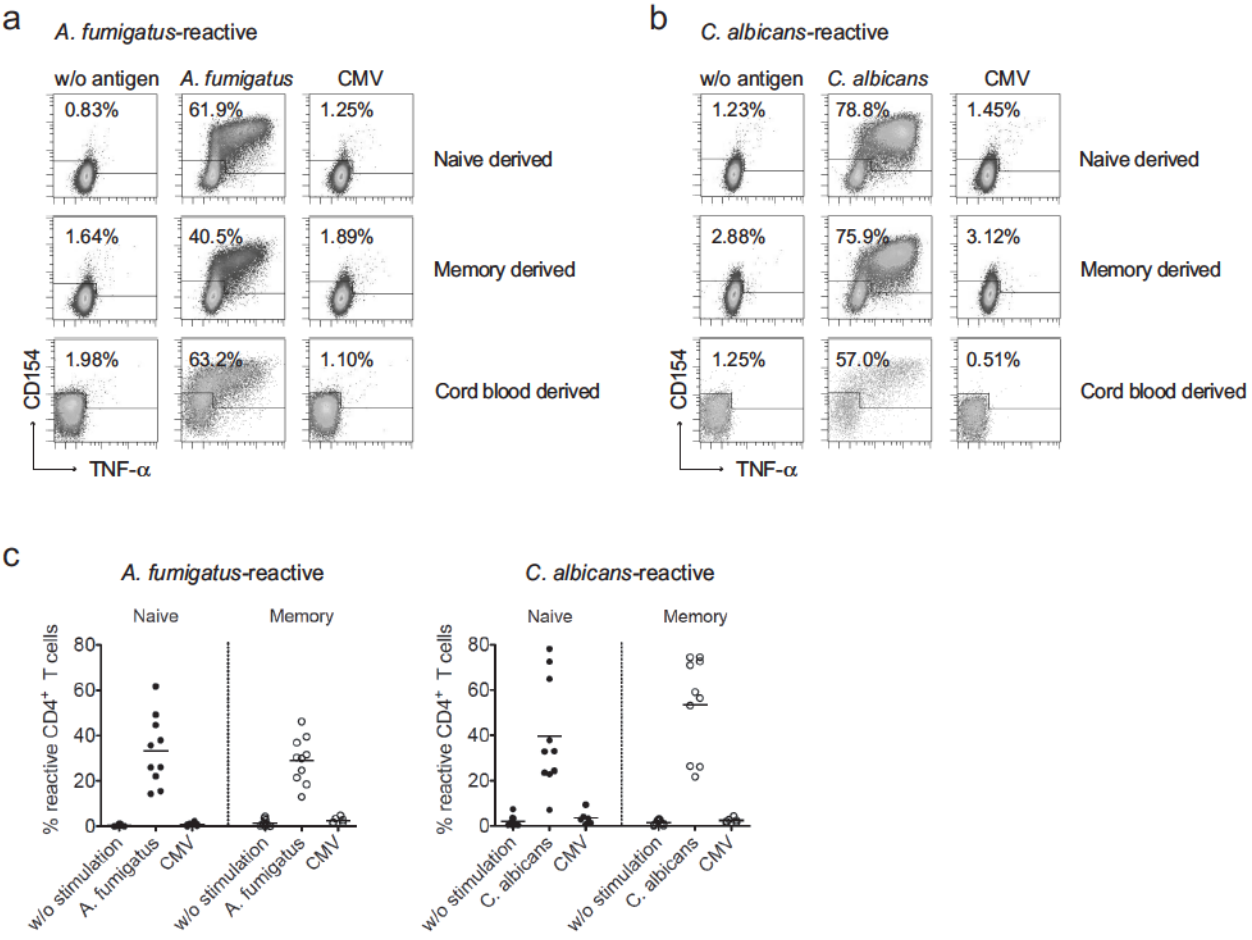

Figure S6. Specificity of fungal lysate stimulated naive and memory CD154$^+$ cells from peripheral blood and cordblood. Pre-sorted memory or naive CD4$^+$ T cells from peripheral blood or cordblood samples were stimulated with (**a**) *A. fumigatus* or (**b**) *C. albicans* lysate. Enriched CD154$^+$ cells were expanded for 2-3 weeks with IL-2 and autologous feeder cells. Expanded cell lines were re-stimulated in presence of autologous APCs with and without antigens as indicated, and reactive CD4$^+$ T cells were determined by CD154 and TNF-α expression. Representative dot plot examples are shown with percentage of reactive cells among total CD4$^+$ T cells and (**c**) summary of several donors with horizontal lines indicating mean values (n = 10, three independent experiments were performed).

Table S1. CF patients characteristics

Patient	Eosinophils [%]	Total IgE [kU/l]	Aspergillus specific IgE		*A. fumigatus*-specific T cell response			
			[kU/l]	Class	% IL-4	% IL-5	% IL-13	Treg/Tmem
non Th2 patients								
1	4.4	187.0	1.3	2	0.84	0.64	1.42	1.93
2	4.3	503.0	8.6	3	1.81	0.79	1.70	2.00
3	0.9	42.9	n.d.	n.d.	1.90	0.21	0.84	0.38
4	7.6	12.0	< 0.15	0	2.16	0.14	0.98	2.27
5	0.6	28.7	< 0.15	0	0.93	n.d.	1.11	2.82
6	1.6	65.9	0.6	1	0.18	n.d.	0.20	0.77
7	5.4	47.2	< 0.15	0	0.00	1.17	0.70	5.10
8	8.4	48.0	< 0.15	0	0.24	0.00	0.53	2.30
Th2 patients								
9	5.4	268.0	15.0	3	27.47	24.77	28.72	n.d.
10	6.8	245.0	22.0	4	13.33	12.03	17.64	0.78
11	4.2	456.0	16.0	3	9.43	5.38	6.92	0.84
12	7.0	159.0	7.6	3	52.56	28.12	54.80	0.91
13	9.6	137.0	46.0	4	12.13	2.88	7.75	0.12
14	2.4	52.0	0.8	2	1.30	0.56	5.91	0.33
15*	3.0	1201.0	40.6	4	13.04	5.73	11.97	0.58
16	7.0	369.0	81.0	5	13.24	8.26	15.53	0.51

Determination of clinical parameters can deviate ± 1 month from time point of blood sampling for T cell analysis.

*Patient 15 was diagnosed for APBA and recieved corticosteroid therapy two month before blood sampling;

n.d. = not determined; Treg = regulatory T cell; Tmem = conventional memory T cell

3.4 Manuskript IV

"The Human Th17 Cell Pool against Airborne Fungal Pathogens is Regulated by Cross-reactivity to Fungal Gut Microbiota"

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The human Th17 cell pool against against airborne fungal pathogens is regulated by cross-reactivity to fungal gut microbiota

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Abstract

Th17 cells are considered mandatory in mediating protection against fungal pathogens. The gut-associated lymphoid tissues have been demonstrated to be the major site for Th17 induction, which critically depends on the composition of the commensal microbiota. Whether and how also peripheral Th17 responses, for example against airborne fungi, are influenced by the gut microbiota is poorly understood. We show that patients with Crohn´s Disease (CD) display increased T cell frequencies and Th17 cytokine production against the gut commensal fungus *C. albicans*. Strikingly, increased *C. albicans* responses are accompanied by a strong expansion of Th17 cells specific for the airborne fungus *A. fumigatus*, resulting from T cell cross-reactivity between both pathogens. In healthy donors up to 30% of the *A. fumigatus*-specific memory T cells cross-react to *C. albicans* and this cross-reactive fraction is strongly increased in CD patients. Intriguingly, within the *A. fumigatus*-specific repertoire, only the cross-reactive T cells are able to secrete IL-17 and IL-22 and express the Th17 cell specific chemokine receptor pattern $CXCR3^{-}CCR6^{+}CCR4^{+}$, whereas non-cross-reactive T cells have a $CXCR3^{+}$ Th1 phenotype. Similar cross-reactive Th17 responses were identified for other airborne fungi, suggesting that induction of Th17 responses via cross-reactivity to fungal gut microbiota is common in airborne fungus-specific T cell responses.

This quantitative and qualitative modulation of human $CD4^{+}$ T cell responses via cross-reactivity to gut microbiota provides an explanation how local immune reactions in the gut may lead to systemic modulation of gut-distal immune responses.

Introduction

Th17 cells are regarded as prototypic effector cells in anti-fungal immune responses and are in general believed to exert a protective function against fungal pathogens. Indeed Th17 cells specific for *Candida albicans*, a member of the human intestinal and skin microbiota, have been identified in human blood (Acosta-Rodriguez et al., 2007; Bacher et al., 2013; Zielinski et al., 2012) and defects in the human Th17 response directly correlate with increased susceptibility to chronic and invasive *Candida* infections in mice and humans (Conti et al., 2009; Eyerich et al., 2008; Huang et al., 2004; Ma et al., 2008; Milner et al., 2008; Puel et al., 2011). However, inappropriate Th17 responses have also been recognized to drive pathologic inflammatory responses against the inhaled fungal pathogen *Aspergillus fumigatus* (Romani et al., 2008; Zelante et al., 2009; Zelante et al., 2007) and more generally seem to be involved in exacerbation of lung inflammatory diseases, such as severe asthma (Akdis et al., 2012; Cosmi et al., 2011). Thus, the role of Th17 cells in immune response against airborne fungal pathogens or allergens is ambiguous and similarly the potential origin of Th17 cells specific for *A. fumigatus* or other airborne fungi is unknown. In contrast to *C. albicans* no selective Th17 inducing capacity of *A. fumigatus* has been reported and recent analyses suggest that *A. fumigatus*-specific T cells in healthy donors are rather dominated by Th1 cells (Bacher et al., 2013; Chai et al., 2010; Chaudhary et al., 2010; Jolink et al., 2013).

The intestinal commensal microbiota is known to have a profound influence on the development and balance of the host immune system including peripheral differentiation of T cell subsets (Backhed et al., 2005; Macpherson and Harris, 2004). Colonization of germfree mice with commensal bacterial species induces the emergence and/or maintenance of distinct T helper cell subsets (Atarashi et al., 2008; Atarashi et al., 2013; Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Ivanov et al., 2008; Lathrop et al., 2011; Mazmanian et al., 2005; Round and Mazmanian, 2010). In particular the induction of Th17 cells has been demonstrated to critically depend on the presence of certain gut microbiota (Atarashi et al., 2008; Ivanov et al., 2009; Ivanov et al., 2008; Wu et al., 2010). Whether the induction of Th17 cells by intestinal microbiota also modulates gut-distal peripheral immune responses against non-gut associated pathogens is currently not known. Since the microbiota provides a tremendous reservoir of potential antigens, T cell receptor cross-reactivity between microbiota and non-gut associated antigens is likely to occur, but has been difficult to systematically analyze due to the complex composition of the microbiome.

Fungi represent an important, though less well studied part of the murine and the human intestinal microbiota and their role in immunomodulation has recently received more attention (Iliev et al., 2012; Moyes and Naglik, 2012; Scanlan and Marchesi, 2008; Scupham et al., 2006). However, the influence of commensal fungi in modulating human T cell responses is largely unknown.

Using a sensitive technology for the direct analysis of antigen-specific $CD4^+$ T cells (Antigen-specific T cell Enrichment, ARTE (Bacher et al., 2013)) we show here, that patients with intestinal inflammation display increased T cell reactivity and Th17 cytokine production against the gut commensal fungus *C. albicans*. Surprisingly this was accompanied by a parallel increase of specific Th17 cells against the major human airborne fungal pathogen *A. fumigatus*. This effect was mediated by specific expansion of T cells, which are cross-reactive to *C. albicans* and also occur in healthy donors and against several other airborne fungi. Intriguingly only the cross-reactive T cells within the *A. fumigatus* response are able to secrete IL-17 and IL-22. Thus we provide a unique example of a quantitative and qualitatively modulation of systemic T cell reactivity via T cell cross-reactivity to intestinal microbiota. Such a mechanism might in general contribute to the well documented link between gut commensal dysbiosis and systemic inflammatory diseases or autoimmunity.

Results

Patients with Crohn´s disease (CD) display altered T cell responses against the gut commensal *C. albicans* and the airborne fungus *A. fumigatus*

Since *C. albicans* is a prominent member of the human gut microbiota we analyzed, whether the *C. albicans*-reactive $CD4^+$ T cell repertoire is altered in CD patients. Following stimulation with fungal lysates $CD154^+$ expression on $CD4^+$ T cells was analyzed. Interestingly, patients with CD showed indeed a significant increase in the frequencies of *C. albicans*-reactive cells (mean 0.41%, range 0.04-1.34%) compared to healthy donors (mean 0.21, range 0.04-0.50%) (Figure 1A). Surprisingly, also the frequencies of T cells specific for the airborne fungus *A. fumigatus* were significantly increased in peripheral blood of CD patients (mean 0.29%, range 0.04-0.94%) and furthermore strongly correlated with the frequencies of *C. albicans*-reactive $CD4^+$ T cells (Figure 1A, B). By contrast, in the healthy control cohort only a marginal correlation between the frequencies of *A. fumigatus* and *C. albicans*-reactive T cells was observed (Figure 1B).

Th1 and Th17 cells have been implicated to play a major role in anti-fungal immunity. We therefore analyzed the cytokine pattern of the fungus-specific T cells in CD patients and healthy controls following magnetic pre-enrichment and multi-parametric flow-cytometric analysis of $CD154^+$ expressing $CD4^+$ T cells (Bacher et al., 2013). Interestingly, *C. albicans*-reactive T cells from CD patients showed a significantly higher production of IFN-γ, IL-17 and IL-22 than the healthy control donors (Figure 1C and Supplemental Figure 1). However, also the *A. fumigatus*-specific T cell response was significantly altered towards a higher production of the Th17 cytokines IL-17 and IL-22. In contrast, the proportion of IFN-γ producing T cells was not increased (Figure 1C). No differences in the production of IL-10 and the Th2 cytokine IL-4 were observed (data not shown).

In summary these data show that the anti-fungal immune response against the gut commensal *C. albicans*, but surprisingly also the airborne fungus *A. fumigatus*, is strongly altered in CD patients. The elevated frequencies of *C. albicans*-reactive T cells are accompanied by increased production of Th1 and Th17 cytokines. However, for *A. fumigatus*, selectively the Th17 response was modified.

A significant proportion of *A. fumigatus*-specific CD4$^+$ T cells is cross-reactive to *C. albicans* antigens

To further investigate a connection between the increased frequencies of specific T cells against the inhaled fungus *A. fumigatus* and CD, we expanded the fungus-specific CD154$^+$ T cells and analyzed potential T cell cross-reactivity by re-stimulation with each fungal pathogen. Analysis of CD154 and cytokine expression upon re-stimulation revealed, that a proportion of *A. fumigatus*-reactive T cells indeed cross-reacted to *C. albicans* antigens and *vice versa* (Figure 2A, B). However, cross-reactivity was more pronounced within the *A. fumigatus*-specific T cell lines, where it accounted for up to 30% (mean 14.7%, range 1.1-31.9) of the total *A. fumigatus*-reactive T cells (Figure 2C). As expected, neither *A. fumigatus*- nor *C. albicans*-lysates activated CMV-specific T cell lines (Figure 2A), providing evidence that the cross-reactivity specifically occurs between both fungi.

To further confirm the cross-reactivity between both fungi on a single cell level, T cell clones were generated after enrichment or depletion of *C. albicans*-cross-reactive CD154$^+$ cells from *A. fumigatus*-specific T cell lines. The specificity of the expanded clones was analyzed by re-expression of CD154 and cytokines (Figure 2D), or proliferation of the cells upon antigen re-stimulation (data not shown). As shown in figure 2D, the cross-reactive T cell clones reacted highly on both, *A. fumigatus* and *C. albicans* antigens, whereas clones from *C. albicans*-depleted *A. fumigatus* reactive T cells, responded only upon *A. fumigatus* re-stimulation.

To further proof whether the expanded T cell response against *A. fumigatus* in CD patients is indeed the results of cross-reactivity to *C. albicans* antigens, *A. fumigatus*-specific T cell lines were expanded from CD patients with initially high or low response to the fungal antigens. Re-stimulation of the cell lines with *C. albicans* revealed a strongly increase of cross-reactivity in those cell lines generated from CD patients with an initial high response, compared to healthy controls or patients with initially low responses (Figure 2E).

Collectively these data show, that the *A. fumigatus*-reactive T cell pool contains a significant proportion of cells with cross-reactivity to *C. albicans* antigens, which accounts for up to 30% of the total *A. fumigatus*-reactive T cell response in healthy donors. In case of an augmented *C. albicans*-response like in CD patients, the expansion of these cross-reactive T cells strongly boosts the response against *A. fumigatus* and cross-reactive cells can constitute up to 60% of the *A. fumigatus*-reactive T cell response.

Cross-reactivity to *C. albicans* induces an IL-17/IL-22-producing subset into the *A. fumigatus*-reactive T cell response

The *A. fumigatus*-reactive $CD4^+$ T cells from CD patients displayed selective alteration in Th17 cytokine production, *i.e.* IL-17 and IL-22 (Figure 1). We therefore investigated the cytokine production of the cross-reactive cells in healthy donors, following re-stimulation of *A. fumigatus*-specific T cell lines. Importantly, T cells with cross-reactivity to *C. albicans* contained strongly increased frequencies of IL-17 and IL-22 producers, whereas TNF-α and IFN-γ production was lower as compared to re-stimulation with *A. fumigatus* lysate (Figure 3A, B). This suggests that the cross-reactive cells selectively have a Th17 phenotype and that a large part of the *A. fumigatus*-specific IL-17 and IL-22 production might be induced by cross-reactivity to *C. albicans*.

To further confirm, that *C. albicans* cross-reactive T cells are enriched in the *A. fumigatus* IL-17/IL-22 producing subset, specific T cell lines were expanded following selection for total $CD154^+$ cells, as well as IFN-γ^+ or IL-17^+/IL-22^+ expressing cells. The *ex vivo* isolated *C. albicans*- and *A. fumigatus*-specific IL-17 producers displayed classical features of Th17 cells, *i.e.* expression of the chemokine receptors CCR6 and CCR4, but not CXCR3 (Figure 3C). In contrast, the majority of *A. fumigatus*-reactive $CD154^+$ memory cells belonged to the $CXCR3^+CCR6^{+/-}$ Th1 subset (Figure 3C). Re-stimulation of the expanded cell lines revealed a strong increase of cross-reactive cells in cell lines selected for IL-17^+/IL-22^+ expression and a significantly decrease in cell lines selected for IFN-γ^+ expression, as compared to the $CD154^+$ enriched cell lines, which comprise the total repertoire of fungus-reactive T cells (Figure 3D, E). In addition, although cross-reactive T cells were only slightly detectable within *C. albicans*-reactive cell lines generated via CD154 selection (Figure 2A, B), a significant increase of cross-reactive cells could be detected in *C. albicans*-reactive cell lines selected for IL-17^+/IL-22^+ production (Figure 3E). These data demonstrate, that *A. fumigatus*/*C. albicans* cross-reactive T cells can be enriched via sorting for IL-17^+/IL-22^+ producers.

To further determine the contribution of cross-reactive T cells to the IL-17^+/IL-22^+ production within the total *A. fumigatus* T cell pool, the *C. albicans*-cross-reactive cells were depleted from the *A. fumigatus*-reactive T cell lines and the remaining *A. fumigatus*-reactive cells were further analyzed for cytokine production. Depletion of the cross-reactive cells after expansion was confirmed by a complete loss of *C. albicans*-reactivity in the depleted *A. fumigatus*-reactive cell lines (Supplemental Figure 2). Importantly, the depletion of cross-reactive cells resulted in a strong decrease of IL-17 and IL-22 expression after re-stimulation with

A. fumigatus-lysate (Figure 3F), indicating that in fact most of the IL-17/IL-22 producing *A. fumigatus*-reactive T cells are cross-reactive to *C. albicans* antigens.

Taken together, these data show that the physiological T cell response against *A. fumigatus* is biased towards a Th1 phenotype, but a significant fraction of *A. fumigatus*-specific T cells cross-reacts to *C. albicans* leading to the selective induction of IL-17^+/IL-22^+ producers. This finding is further supported by a positive correlation between the *ex vivo* frequencies of *C. albicans*-specific T cells and *A. fumigatus*-specific IL-17 producers, as well as between the frequency of *C. albicans*- and *A. fumigatus*-specific IL-17 producers (Figure 3G).

C. albicans-cross-reactive T cells are selectively expanded within the *A. fumigatus*-reactive memory $CD4^+$ T cell pool

We next analyzed, whether the cross-reactivity is the result of a natural overlap in the TCR repertoire due to protein sequence similarities between both fungal species or whether it reflects an active modulation of the response to *A. fumigatus* by selective expansion of cross-reactive T cells *in vivo*. Cross-reactivity was mainly observed within the *A. fumigatus*-reactive T cell lines but was significant lower within *C. albicans*-reactive T cell lines (Figure 2), arguing against a mutual overlap of the TCR repertoire. To further corroborate this point, *A. fumigatus*-reactive T cell lines were generated from pre-sorted naive and memory T cells and were re-stimulated with lysates of different fungal pathogens. As shown in Figure 4A, *A. fumigatus*-reactive cell lines from the naive and memory compartment reacted also highly upon stimulation with other *Aspergillus* species, indicating high cross-reactivity due to protein sequence similarities between closely related fungi. In contrast, the cross-reactivity to more distantly related fungal species was absent in cell lines from both compartments. Importantly, whereas *A. fumigatus*-reactive T cells generated from memory T cells showed high cross-reactivity to *C. albicans*, there was no cross-reactivity above background detectable in T cells generated from the naive T cell pool (Figure 4A, B). In addition, contrary to Th1 cytokines, which were strongly induced also in naive-derived T cells, the production of IL-17 and IL-22 was restricted to T cells generated from the memory T cell pool (Figure 4B). This rules out the possibility that the IL-17^+/IL-22^+ producing cells were induced under the experimental conditions during *in vitro* culture.

In summary, the presence of cross-reactive T cells exclusively in the memory pool further emphasizes that *C. albicans* promotes the selective expansion of cross-reactive T cells with IL-17$^+$/IL-22$^+$ producing capacities within the *A. fumigatus*-specific memory T cell repertoire.

Airborne fungal Th17 responses in humans result from cross-reactivity to *C. albicans*

To investigate a potential general influence of *C. albicans*-driven cross-reactivity on the T cell response against airborne fungi, we further analyzed the T cell response against 10 other human respiratory fungal pathogens. Importantly, the *ex vivo* analysis of enriched CD154$^+$ T cells revealed, that only against *C. albicans* IL-17 was the dominating cytokine. In contrast, against all other fungal pathogens, only a minor proportion of the CD154$^+$ cells produced IL-17, and IFN-γ expression was prevailing (Figure 5 A, B). Furthermore, re-stimulation of expanded fungus-specific T cells lines showed in each case a higher IL-17 production of the few *C. albicans* cross-reactive cells, than following re-stimulation with the specific fungal antigens (Figure 5C). These data suggests that also in the response against the other fungal pathogens, a significant proportion of the IL-17 producing T cells are induced via cross-reactivity to *C. albicans* antigens. To further confirm this finding, the *ex vivo* IL-17 secretion of fungus-specific T cells was analyzed following depletion of *C. albicans*-reactive CD154$^+$ cells from PBMCs. The depletion resulted in a complete loss of *C. albicans* reactive T cells (Figure 5D), and importantly, IL-17 production of the fungus-specific T cells was strongly reduced in *C. albicans*-depleted *versus* non-depleted PBMC (Figure 5E).

Discussion

We provide a unique example how gut commensal microbiota can influence the immune response against non-gut associated peripheral antigens, through the selective expansion of particular T helper cell subsets that would not naturally develop in response to the peripheral antigen. Furthermore we show that this effect is further exacerbated in CD patients, which may in general contribute to the well-known systemic manifestations of intestinal pathology and dysbiosis of the microbiota. The commensal microbiota is known to have a strong influence on the development of the human immune system as well as on local and systemic immunopathologies (Macpherson and Harris, 2004). However, to what extent cross-reactivity between microbiota and peripheral antigens modulates specific T cell responses is less well studied. Specific examples are rare due to the antigenic complexity in physiological systems such as the human immune system and the resulting difficulties to analyze the rare antigen-specific T cells and in particular $CD4^+$ T cells.

The phenomenon of cross-reactivity caused by heterologous immunity and/or molecular mimicry is well established in experimental model systems (Selin et al., 2006; Welsh et al., 2010; Welsh and Selin, 2002). So far it has mainly been described for viral antigens and in particular for $CD8^+$ T cells (Su and Davis, 2013; Welsh et al., 2010) although a few examples for virus-specific $CD4^+$ T cells have also been described (Roti et al., 2008; Su et al., 2013; Wucherpfennig and Strominger, 1995). These studies show, that cross-reactivity can have marked consequences on the outcome of a subsequent antigen challenge, by altering hierarchies and maintenance of the T cell repertoire, which might lead to either enhanced or diminished protection or even immunopathology (Brehm et al., 2002; Chen et al., 2001; Selin et al., 1994; Selin et al., 1998; Welsh et al., 2010), *e.g.* by cross-reactivity to auto- (McCoy et al., 2006; Sospedra et al., 2005; Wucherpfennig and Strominger, 1995) or allo-antigens (Burrows et al., 1994; D'Orsogna et al., 2010). Since the commensal microbiota represents an extraordinary large source of antigens, it can be expected to engage a significant fraction of the human T cell repertoire (Belkaid et al., 2013). As a consequence, cross-reactivity driven by commensal microbiota might be a common event in shaping antigen-specific T cell responses against non-related antigens. In line with this idea, it has recently been shown, that virus-specific memory $CD4^+$ T cells are abundant in unexposed donors, and show cross-reactivity to commensal bacterial peptides (Su et al., 2013). However, to our knowledge specific examples for the antigen-specific functional modulation of systemic human $CD4^+$ T

cell responses by a specific member of the intestinal microbiota in health and during intestinal inflammation are so far not described.

We show that T cell cross reactivity triggered by the common gut commensal *C. albicans* has a profound influence on the human immune response against airborne fungal pathogens, by the selective induction of Th17 cells in an otherwise Th1 biased immune response. These cross-reactive T cells remarkably accounted for up to one third of the memory T cell pool against the constantly inhaled opportunistic fungus *A. fumigatus* in healthy donors. During gut inflammation in Crohn´s disease, a subset of patients revealed elevated responsiveness against *C. albicans* probably as a result of increased access to intestinal luminal antigens due to a reduced barrier function of the intestinal wall (Duchmann et al., 1999; Peeters et al., 1997; Soderholm et al., 1999). Strikingly, in this pathological situation the expansion of cross-reactive memory T cells strongly boosts the response against *A. fumigatus*. In contrast, within the *C. albicans*-reactive T cell pool, cross-reactive T cells against *A. fumigatus* were underrepresented, indicating that these cells are not the result of a natural overlap between the specific T cell repertoires against both fungi due to a high number of conserved antigenic epitopes. This is also confirmed by our finding that cross-reactive cells were only found within the *A. fumigatus*-specific memory but not the naive T cell pool, indicating that a selective expansion of cross-reactive cells, and at the same time depletion from the naive repertoire, occurs *in vivo*.

Intriguingly, the *C. albicans* driven cross-reactive T cells had a clear Th17 phenotype ($CXCR3^{-}CCR6^{+}CCR4^{+}$; production of IL-17 and IL-22, but no IFN-γ), which is clearly distinguished from the Th1 phenotype of the non-cross-reactive *A. fumigatus*-specific T cells. Several studies have highlighted a critical role for gut microbiota in the differentiation of Th17 cells in the lamia propria (Atarashi et al., 2008; Ivanov et al., 2009; Ivanov et al., 2008; Wu et al., 2010). Indeed, at steady state, Th17 cells are most abundant in gut-associated tissues, whereas only a very small proportion of $CD4^{+}$ T cells express IL-17 in extra-intestinal sites (Atarashi et al., 2008; Ivanov et al., 2008; Ivanov et al., 2006). However, whether these Th17 cells in the gut are specific to antigens of the commensal microbiota or may also recognize other gut-distal antigens has so far not been demonstrated. Consistent with the link of Th17 cells to gut microbiota, we found that the human T cell response against all analyzed inhaled fungal pathogens contains in contrast to *C. albicans* only a minor population of IL-17 producing T cells. However, a small Th17 population was consistently present in the T cell response against all airborne fungi we analyzed. Depletion of *C. albicans*-reactive T cells confirmed, that virtually all of these Th17 cells specific for inhaled fungi are cross-reactive to

C. albicans. The IL-17 and IL-22 producers had a CD45RO memory phenotype and were not the result of an *in vitro* induction process, since we found them *in vitro* exclusively in memory-derived T cell lines but not in expanded T cell lines derived from the naive pool. These data implicate that a small number of cross-reactive precursors is selectively expanded and driven into Th17 lineage differentiation upon *C. albicans* encounter and in this way induces a new Th cell subset in the specific T cell response against inhaled fungal antigens. This is further supported by our finding that the *ex vivo* frequencies of *A. fumigatus* and *C. albicans*-specific $CD154^+$ T cells in healthy donors only weakly correlated. Instead, we observed a strong positive correlation between the frequencies of *A. fumigatus*-specific IL-17 producers and total *C. albicans*-reactive T cells, as well as the frequency of *C. albicans*-specific IL-17 producers. Thus interaction of the immune system with *C. albicans* seems to have a major impact on the specific T cell response against *A. fumigatus*, as well as on other airborne fungi, by shaping and expanding a population of cross-reactive T cells with a unique Th17 phenotype not found in the natural response to these antigens alone. This provides a new mechanism of how T cell cross-reactivity can have marked consequences on the outcome of immune responses by the induction of a functionally unique Th cell subset.

In contrast to *C. albicans* infection, where a protective role of Th17 cells is well established (McDonald, 2012), the role of Th17 cells against inhaled fungal pathogens is a matter of debate. Protective roles for IL-17 and Th17 cells have been described in mouse models for different fungal pathogens, such as *Cryptococcus neoformans*, *Pneumocystis carinii* or *Blastomyces dermatitidis* (Kleinschek et al., 2006; Rudner et al., 2007; Wüthrich et al., 2013). However, other studies suggest that Th17 cells exacerbate inflammation and induce severe tissue pathology in mouse models of pulmonary aspergillosis (Romani et al., 2008; Zelante et al., 2009; Zelante et al., 2007). Recently it has been shown that IL-17 drives Th2-type allergic pulmonary eosinophilia following repeated inhalation of *A. fumigatus* conidia (Murdock et al., 2012). In line with these data, in particular Th17 responses have been linked to a variety of different airway disorders, including asthma, inflammatory lung disease and airway hypersensitivity (Akdis et al., 2012; Cosmi et al., 2011). Thus, the selective induction of Th17 cells into the immune response against inhaled fungal pathogens by *C. albicans* cross-reactivity might even have pathologic consequences by driving allergic lung diseases against fungi, following sensitization to inhaled fungal antigens.

Strikingly, in some patients with Crohn´s disease, *C. albicans*-specific T cell frequencies were strongly increased and expanded cross-reactive T cells quantitatively and qualitatively modulated the immune response against *A. fumigatus*. Regarding these results it is interesting

to note that an increased incidence of asthma has been reported in patients with Crohn´s disease (Bernstein et al., 2005; Spira et al., 1998) and perturbations of the microbiota composition have been correlated to the development of allergic diseases (Bottcher et al., 2002; Goldman and Huffnagle, 2009; Kalliomaki et al., 2001; Kirjavainen et al., 2002). Thus, the modulation of specific T cell responses against normally harmless environmental antigens by cross-reactivity against gut microbiota might be a broadly relevant event during gut inflammation and might contribute to the effect of gut microbiota on distantly located immune responses and systemic immunopathology.

Materials and Methods

Blood donors

Buffy coats from healthy donors were obtained from the university hospital in Dortmund. Peripheral ethylenediaminetetraacetic acid (EDTA) blood samples were obtained from healthy volunteers and patients with Crohn´s disease (CD). The study was performed according to established ethical guidelines (Ethical Comittee of Charité – University Medicine Berlin). All human blood donors gave informed consent.

Peripheral blood mononuclear cells (PBMCs) were separated by use of Ficoll-Hypaque (GE Healthcare Life Sciences, Freiburg, Germany) density gradient centrifugation and were resuspended in RPMI-1640 (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with 5% (v/v) inactivated human AB-serum (BioWhittaker/Lonza, Walkersville, MD, USA) and 2mM L-glutamine (PAA Laboratories, Pasching, Austria).

Fungal lysates

The *Aspergillus fumigatus* strain ATCC 46645 (LGC Standars, Wesel, Germany), the *A. niger* strain FSU871, the *A. nidulans* strain RMSO11 and *A. terreus* strain i402 were used for preparation of protein extracts from mycelia. Conidia were inoculated at a final concentration of $2x10^6$ spores/ml in YPD or AMM medium and shaken at 37 °C or 30 °C (for *A. niger*) with 200 rpm. After 20 hours of incubation, mycelia were recovered by filtration and disrupted in a microdismembrator (Sartorius Stedim Biotech GmbH, Göttingen, Germany) at 200 rpm for 10 min using 0.5 mm glass beads. Clinical isolates of *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, *Lichtheimia corymbifera, Rhizopus oryzae, Scedosporium apiospermum,* and *S. prolificans* obtained from the Fungiscope™ study were grown on Sabouraud dextrose agar plates until sporulation. Conidia were shaken in RPMI-1640 medium supplemented with MOPS (3-(N-morpholino) propane sulfonic acid), glutamine and 2 % glucose for 5-7 days at 37° C. Mycelia were disrupted using the gentleMACS device (Miltenyi Biotec) program RNA.01 for 5 times. All lysates were resuspended in 1×PBS buffer (2 mM $MgCl_2$), and centrifuged for 20 min at 20,000 × g. Supernatants were stored in aliquots at -20 °C until use. The *Candida albicans* lysate was purchased from Greer Laboratories, Lenoir, NC, USA.

Stimulation of human PBMCs and antigen-reactive T cell enrichment (ARTE)

$1×10^7$ PBMCs were stimulated for 7 hours with 40 µg/ml of the fungal lysates in presence of 1 µg/ml CD40 and 1 µg/ml CD28 pure antibody (both Miltenyi Biotec). 1 µg/ml Brefeldin A

(Sigma-Aldrich, Schnelldorf, Germany) was added for the last 2 hours. Cells were separated using the CD154 MicroBead Kit (Miltenyi Biotec). In brief, cells were indirectly magnetically labeled with CD154-Biotin and anti-Biotin microbeads and enriched by two sequentially MS columns (Miltenyi Biotec). For characterization of phenotypic markers, surface staining was performed on the first column, followed by fixation, permeabilization (Inside stain Kit; Miltenyi Biotec) and intracellular cytokine staining on the second column, as described (Bacher et al., 2013).

Flow cytometric analysis

Depending on the experiment, cells were stained in different combinations with following mAbs (clone names in parantheses): CD4-VioBlue, CD4-APC-Vio770 (VIT4); CD45RO-PE-Vio770 (UCHL1); CD8-PerCP, CD8-VioGreen (BW135/80); CD14-PerCP, CD14-VioGreen (TÜK4); CD20-PerCP, CD20-VioGreen (LT20); CD154-VioBlue, CD154-APC (4E3); TNF-α-FITC (cA2); IFN-γ-APC, IFN-γ-FITC, IFN-γ-PE (45-15); IL-17A-APC, IL-17A-FITC, IL-17A-PE-Vio770 (CZ8-23G1); IL-10-PE (JES3-9D7), CXCR3-PE-Vio770 (REA232) (all from Miltenyi Biotec); IL-4-APC (7A3-3) (conjugated in-house); CCR6-BV421 (G034E3); CCR4-PerCP-Cy5.5 (TG6); IFN-γ-PerCP-Cy5.5 (4S.B3) (all from BioLegend, San Diego, CA, USA); IL-22-PerCP-Cy5.5 (22URTI) (from eBioscience, San Diego, CA, USA); IL-22-PE (142928) (from R&D systems). Data were acquired on a MACSQuant analyzer and MACSQuantify software (both Miltenyi Biotec) was used for analysis.

Expansion of fungus-specific T cell lines

Magnetic enrichment of $CD154^+$ cells was performed using the CD154 MicroBead Kit and cytokine secreting cells were isolated using the IFN-γ-PE and IL-17A-APC Secretion Assay-Cell Enrichment and Detection Kit according to the manufacturer´s instructions (all from Miltenyi Biotec). The IL-22 secretion assay was conjugated in-house.

For expansion of T cell lines from the naive or memory repertoire, $CD45RA^+$ or $CD45RO^+$ $CD4^+$ T cells were purified from PBMC by negative selection using the Naive $CD4^+$ T Cell Isolation Kit II or the Memory $CD4^+$ T Cell Isolation Kit (both Miltenyi Biotec), respectively. For stimulation of the isolated naive and memory cells, CD3-depleted APCs were generated using CD3 MicroBeads and LD columns (both Miltenyi Biotec) and added in a ratio of 1:1. Alternatively, if only cells from the memory compartment were expanded, $CD45RA^+$ cells were depleted from PBMC prior stimulation using CD45RA Microbeads and LD columns (both Miltenyi Biotec). $CD154^+$ cells were enriched as described above.

Enriched fungus-specific T cells were expanded with mitomycin C (Sigma Aldrich) treated autologous feeder cells in a ratio of 1:100 at a density of 2.5×10^6 cells/cm^2 in 48-well plates in X-Vivo15 (BioWhittaker/Lonza, Walkersville, MD, USA), supplemented with 5% (v/v) AB-serum (BioWhittaker/Lonza), 200 U/ml IL-2 (Proleukin®; Novartis, Nürnberg, Germany) and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Antibiotic Antimycotic Solution, Sigma Aldrich). Cells were expanded for 14-21 days, culture medium was replenished and cells were split, when needed.

Two days before re-stimulation, expanded cells were rested in RPMI-1640 + 5% (v/v) human AB-serum without IL-2. 5×10^5 expanded T cells were re-stimulated with autologous CD3-depleted PBMC as APCs in a ratio of 1:1 and 40µg/ml fungal lysate or 10µg/ml CMV lysate (Siemens Healthcare Diagnostics, Marburg, Germany) and 1µg/ml CD28 functional grade pure Ab for 3h plus additionally 4h with 1µg/ml Brefeldin A (Sigma Aldrich). After fixation and permeabilization cells were stained intracellularly for CD154 and cytokine expression.

Generation of specific T cell clones

Expanded *A. fumigatus*-specific T cell lines were re-stimulated with autologous CD3-depleted APCs and *C. albicans* for 7 hours in presence of 1 µg/ml CD40 mAb (Miltenyi Biotec). Cross-reactive CD154$^+$ T cells were isolated using CD154-PE, anti-PE MicroBeads and 2 MS columns (all Miltenyi Biotec). From the negative fraction, CD4$^+$ T cells were purified using an untouched isolation with the CD4$^+$ T cell isolation Kit II (Miltenyi Biotec) and stimulated with autologous CD3-depleted APCs and *A. fumigatus* for 7 hours in presence of 1µg/ml CD40 mAb (Miltenyi Biotec). *C. albicans*-depleted *A. fumigatus*-reactive CD4$^+$ T cells were isolated using CD154-PE, anti-PE MicroBeads and 2 MS columns (all Miltenyi Biotec). Cross-reactive and *C. albicans* depleted T cells were cloned by limiting dilution by seeding 1 cell/well into 96-well round bottom plates together with 5×10^4/well mitomycin C (Sigma Aldrich) treated feeder cells and 200 µl X-Vivo15 (BioWhittaker/Lonza), supplemented with 10% (v/v) AB-serum (BioWhittaker/Lonza), 200 U/ml IL-2 (Proleukin®; Novartis) and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Antibiotic Antimycotic Solution, Sigma Aldrich). Clones were stimulated with 1 µg/ml PHA (Sigma Aldrich) and expanded for 4 weeks with 5×10^4/well feeder cells and 1 µg/ml PHA added on day 10.

Re-stimulation was performed by stimulating 5×10^5 expanded T cell clones/well with 5×10^5 autologous CD3-depleted APCs with or without antigen for 7 hours. 1µg/ml Brefeldin A was added for the last 4 hours. Specificity of the expanded T cell clones was analyzed by

intracellular staining for CD154 and cytokines or proliferation on day 7 after labeling with CellTrace™ Violet Cell Proliferation Kit (Invitrogen™, Molecular Probes®, Eugene, OR, USA).

Depletion of *C. albicans* cross-reactive T cells

For depletion of *C. albicans* cross-reactive cells from *A. fumigatus*-reactive T cell lines, 5×10^6 *A. fumigatus*-reactive T cells were stimulated with 5×10^6 autologous CD3-depleted APCs and *C. albicans*, in presence of 1 µg/ml CD28 and 1 µg/ml CD40 mAb (Miltenyi Biotec). After 7 hours of stimulation, $CD154^+$ cells were depleted, using the CD154 MicroBead Kit and a single MS column (Miltenyi Biotec). $CD4^+$ T cells were isolated from the negative fraction, using an untouched isolation with the $CD4^+$ T cell isolation Kit II (Miltenyi Biotec). The remaining *C. albicans*-depleted *A. fumigatus*-reactive $CD4^+$ T cells were again stimulated with autologous CD3-depleted APCs in a ratio of 1:1 for 7h hours with *A. fumigatus*-lysate and 1µg/ml Brefeldin A added for the last 4 hours and analyzed for CD154 and cytokine expression.

For *ex vivo* depletion of *C. albicans*-reactive T cells, PBMCs were stimulated for 7 hours with *C. albicans* lysate in presence of 1 µg/ml CD28 and 1 µg/ml CD40 mAb (Miltenyi Biotec). *C. albicans*-reactive T cells and non-T cells were depleted by labelling with the untouched $CD4^+$ T cell isolation Kit II and CD154-Biotin, followed by anti-Biotin MicroBeads using LS columns (all Miltenyi Biotec). The remaining *C. albicans* depleted $CD4^+$ T cells were stimulated with autologous CD3-depleted APCs and different fungal lysates for 5 hours plus additionally 2h with 1 µg/ml Brefeldin A (Sigma Aldrich). ARTE was performed as described above.

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Figure legends

Figure 1. Altered fungus-specific T cell response in patients with Crohn´s disease (CD)
(A) Frequencies of *C. albicans*- and *A. fumigatus*-reactive $CD154^+$ T cells in PBMCs of healthy donors (n=111) *versus* patients with Crohn´s disease (n=46). A cut-off value ≥ 0.4% $CD154^+$ *C. albicans*-reactive T cells was defined to separate patients with (red symbols) and without (blue symbols) increased T cell reactivity. (B) Correlation of $CD154^+$ frequencies of *C. albicans*- with *A. fumigatus*-reactive T cells in CD patients. (C) *Ex vivo* analysis of cytokine production of *A. fumigatus*- and *C. albicans*-reactive T cells following $CD154^+$ enrichment. Percentages of cytokine producing cells among $CD154^+$ cells are shown in CD patients and healthy controls. Significance was determined using unpaired Student´s *t*-test and Pearson´s correlation coefficient was used to calculate correlations.

Figure 2. *A. fumigatus*-reactive T cell lines contain cells cross-reactive to *C. albicans*.
(A, B) PBMCs of CMV sero-positive donors were stimulated for 7 hours with *A. fumigatus*-, *C. albicans*- or CMV-lysate. Antigen-reactive $CD154^+$ cells were isolated and subsequently expanded for 14 days with IL-2 and autologous feeder cells. Expanded cell lines were re-stimulated in presence of autologous APCs with and without antigens as indicated, and reactive $CD4^+$ T cells were determined by CD154 and TNF-α expression. (A) Representative dot plot examples of one donor and (B) statistical analysis for several donors (n=30). (C) Percentage of cross-reactivity was calculated in relation to total reactivity after re-stimulation with the specific fungal lysate. *p* values were determined using paired Student´s *t*-test. (D) Single T cell clones were generated from enriched cross-reactive $CD154^+$ cells or after depletion of cross-reactive cells from *A. fumigatus*-reactive T cell lines. Reactivity was determined by expression of CD154 and cytokines upon re-stimulation. (E) *A. fumigatus*-reactive T cell lines were expanded following $CD154^+$ enrichment from healthy donors or CD patients with high or low frequencies of reactive T cells. Cells were re-stimulated with *C. albicans* lysate and percentage of cross-reactivity is shown in relation to the total reactivity after re-stimulation with *A. fumigatus* lysate. Significance was determined using unpaired Student´s *t*-test.

Figure 3. Cross-reactive T cells produce IL-17 and Il-22. (A, B) *A. fumigatus*-reactive T cell lines were generated, as described in figure 2. Cell lines were re-stimulated either with *A. fumigatus* or *C. albicans* antigens and analyzed for percentages of cytokine producing cells among $CD4^+CD154^+$. (A) Representative dot plot examples and (B) statistical analysis from several donors (n=6). (C) *Ex vivo* enriched *A. fumigatus*- and *C. albicans*-reactive cells were gated as indicated and stained for CXCR3, CCR6 and CCR4 expression. Percentage of positive cells within the respective gates is indicated. (D) *A. fumigatus*-reactive T cell lines were expanded after selection for CD154, IFN-γ producers or IL-17/IL-22 producers and re-stimulated with *A. fumigatus* or *C. albicans* lysate. (E) Percentage of cross-reactivity was calculated in relation to total reactivity after re-stimulation with the specific antigen lysate. (F) *A. fumigatus*-reactive T cell lines generated via CD154-enrichment were re-stimulated with *C. albicans*-lysates and cross-reactive $CD154^+$ cells were depleted. Analysis of IL-17 and IL-22 expression is shown of total *A. fumigatus*-reactive and *C. albicans*-depleted cell lines after re-stimulation with *A. fumigatus* lysate. Dot plot examples for one donor with numbers indicating percentage of cytokine producing cells among $CD4^+CD154^+$ and summary for several donors (n=5) is shown. (G) PBMCs were *ex vivo* stimulated with *A. fumigatus* or *C. albicans* lysates and analyzed for IL-17 production following enrichment of $CD154^+$ T cells. Correlations obtained by linear regression curve analysis are shown for the frequencies of total *C. albicans*-reactive $CD154^+$ T cells versus *A. fumigatus*-specific IL-17 producers (n=57; left diagram) or the frequencies of *A. fumigatus*- versus *C. albicans*-specific IL-17 producers among $CD154^+$ (n=57; right diagram). Pearson´s correlation coefficient was used to calculate correlations. Significance was determined using paired Student´s *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 4. *C. albicans* cross-reactive T cells are expanded within the *A. fumigatus*-reactive memory $CD4^+$ T cell pool. Memory or naive $CD4^+$ T cells were pre-sorted from PBMCs and stimulated with CD3-depleted APCs and *A. fumigatus* lysate. Enriched $CD154^+$ cells were expanded for 14 days with IL-2 and autologous feeder cells. Expanded cell lines were re-stimulated in presence of autologous APCs with different fungal antigen lysates as indicated, and reactive $CD4^+$ T cells were determined by CD154 and TNF-α expression. (A) Percentage of reactive $CD4^+$ T cells of memory derived (left) and naive derived (right) *A. fumigatus*-reactive T cell lines (n=4). (B) Percentage of cross-reactivity to *C. albicans* of the different selected T cell lines was calculated in relation to total reactivity after re-stimulation with

A. fumigatus lysate (n=10). Significance was determined using paired Student´s *t*-test. (C) Frequencies of cytokine producing cells among $CD4^+CD154^+$ of memory derived (left) and naive derived (right) *A. fumigatus*-reactive T cell lines re-stimulated with *A. fumigatus* or *C. albicans* lysate (n=6).

Figure 5. IL-17 producers in airborne fungus-reactive T cell responses are cross-reactive to *C. albicans*. (A, B) *Ex vivo* analysis of cytokine production of reactive T cells against various fungal lysates is shown following $CD154^+$ enrichment. Percentages of cytokine producing cells among $CD154^+$ cells are indicated. (A) Representative dot plots and (B) statistical analysis for several donors. (C) CD45RA-depleted PBMCs were stimulated with the indicated fungal lysates, $CD154^+$ T cells were expanded and re-stimulated either with the specific antigen or *C. albicans*. Percentage of Il-17 producing cells among CD154 is shown. Graph represents mean values ± SEM from 5-6 donors. (D) PBMCs were stimulated with *C. albicans* and reactive $CD154^+$ cells were depleted. Frequency of *C. albicans*-specific $CD154^+$ cells prior and after depletion is shown. (E) PBMCs and *C. albicans*-depleted PBMCs were *ex vivo* stimulated with the indicated fungal lysates and percentage of IL-17 producers among $CD154^+$ is shown.

Figure 1

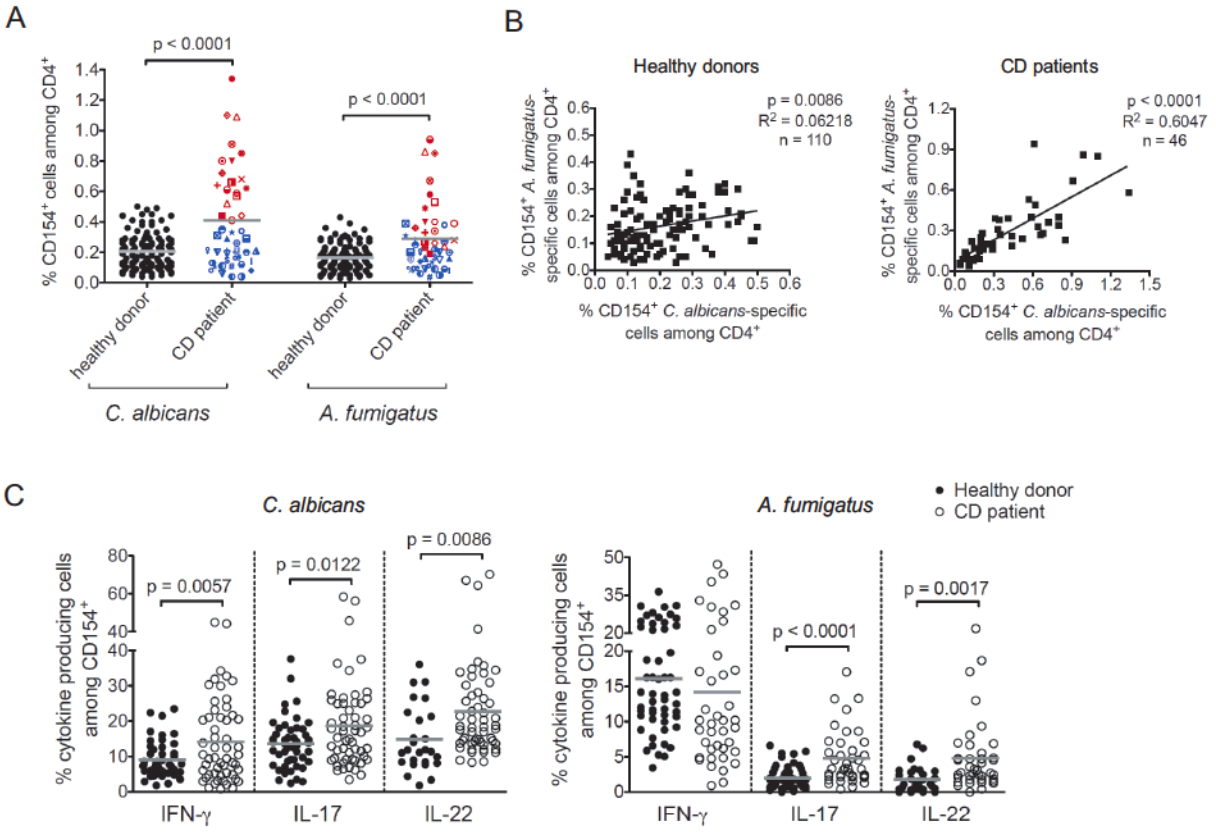
A
p < 0.0001
p < 0.0001
% CD154+ cells among CD4+
healthy donor
CD patient
healthy donor
CD patient
C. albicans
A. fumigatus
B
Healthy donors
p = 0.0086
R2 = 0.06218
n = 110
% CD154+ A. fumigatus-specific cells among CD4+
% CD154+ C. albicans-specific cells among CD4+
CD patients
p < 0.0001
R2 = 0.6047
n = 46
% CD154+ A. fumigatus-specific cells among CD4+
% CD154+ C. albicans-specific cells among CD4+
C
C. albicans
A. fumigatus
Healthy donor
CD patient
% cytokine producing cells among CD154+
p = 0.0057
p = 0.0122
p = 0.0086
IFN-γ
IL-17
IL-22
% cytokine producing cells among CD154+
p < 0.0001
p = 0.0017
IFN-γ
IL-17
IL-22

Figure 2

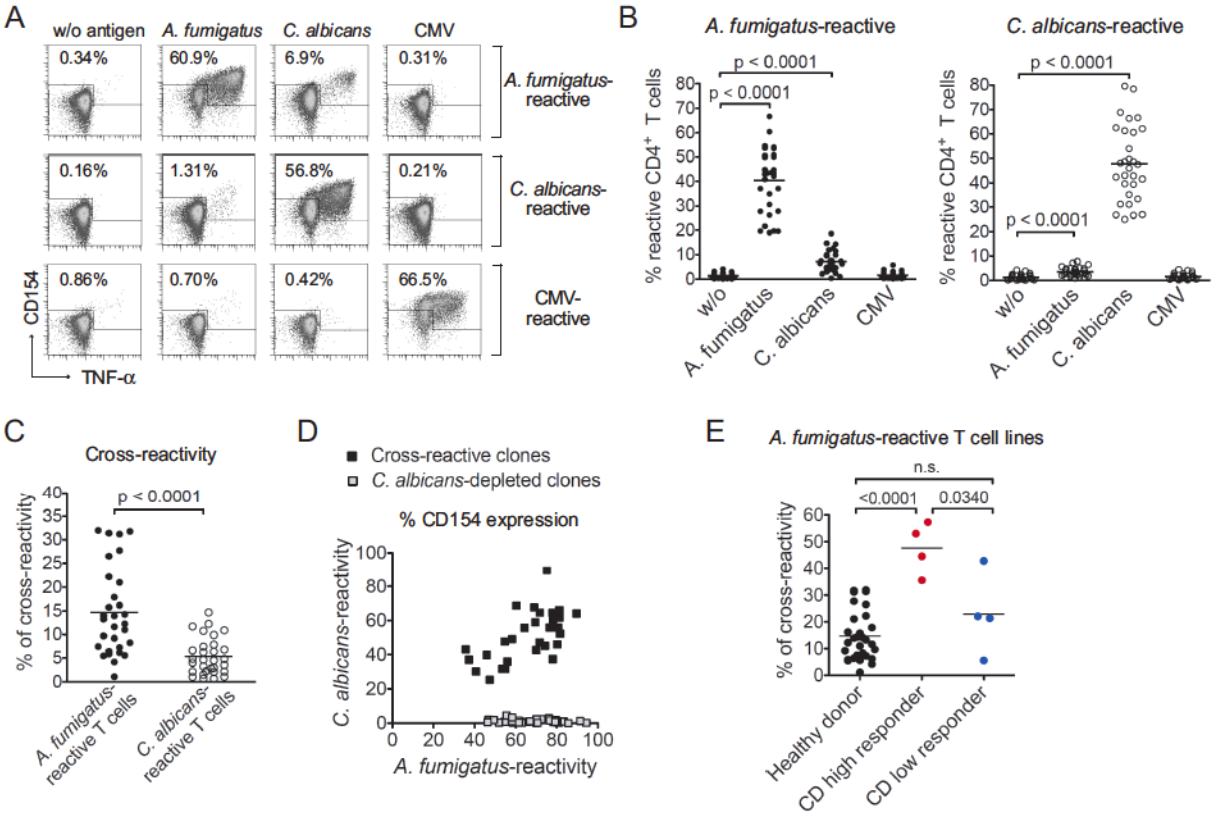
A
w/o antigen
A. fumigatus
C. albicans
CMV
0.34%
60.9%
6.9%
0.31%
A. fumigatus-reactive
0.16%
1.31%
56.8%
0.21%
C. albicans-reactive
0.86%
0.70%
0.42%
66.5%
CMV-reactive
CD154
TNF-α
B
A. fumigatus-reactive
C. albicans-reactive
% reactive CD4+ T cells
p < 0.0001
w/o
A. fumigatus
C. albicans
CMV
C
Cross-reactivity
% of cross-reactivity
A. fumigatus-reactive T cells
C. albicans-reactive T cells
D
Cross-reactive clones
C. albicans-depleted clones
% CD154 expression
C. albicans-reactivity
A. fumigatus-reactivity
E
A. fumigatus-reactive T cell lines
n.s.
<0.0001
0.0340
Healthy donor
CD high responder
CD low responder

Figure 3

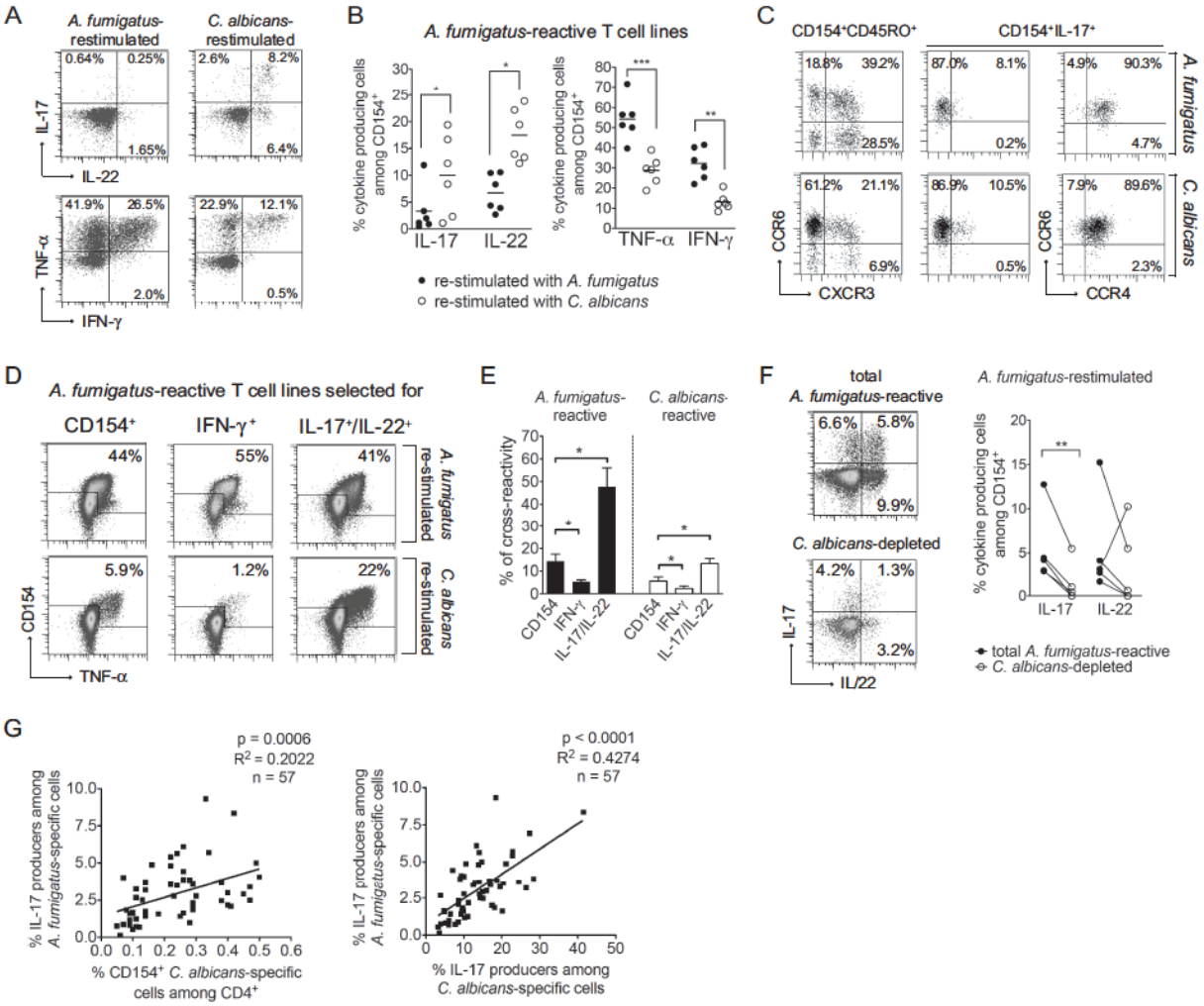
A
A. fumigatus-restimulated
C. albicans-restimulated
IL-17
IL-22
TNF-α
IFN-γ
B
A. fumigatus-reactive T cell lines
% cytokine producing cells among CD154+
IL-17
IL-22
TNF-α
IFN-γ
re-stimulated with A. fumigatus
re-stimulated with C. albicans
C
CD154+CD45RO+
CD154+IL-17+
A. fumigatus
C. albicans
CCR6
CXCR3
CCR4
D
A. fumigatus-reactive T cell lines selected for
CD154+
IFN-γ+
IL-17+/IL-22+
A. fumigatus re-stimulated
C. albicans re-stimulated
CD154
TNF-α
E
A. fumigatus-reactive
C. albicans-reactive
% of cross-reactivity
CD154
IFN-γ
IL-17/IL-22
F
total A. fumigatus-reactive
A. fumigatus-restimulated
C. albicans-depleted
IL-17
IL22
% cytokine producing cells among CD154+
total A. fumigatus-reactive
C. albicans-depleted
G
p = 0.0006
R2 = 0.2022
n = 57
% IL-17 producers among A. fumigatus-specific cells
% CD154+ C. albicans-specific cells among CD4+
p < 0.0001
R2 = 0.4274
n = 57
% IL-17 producers among C. albicans-specific cells

Figure 4

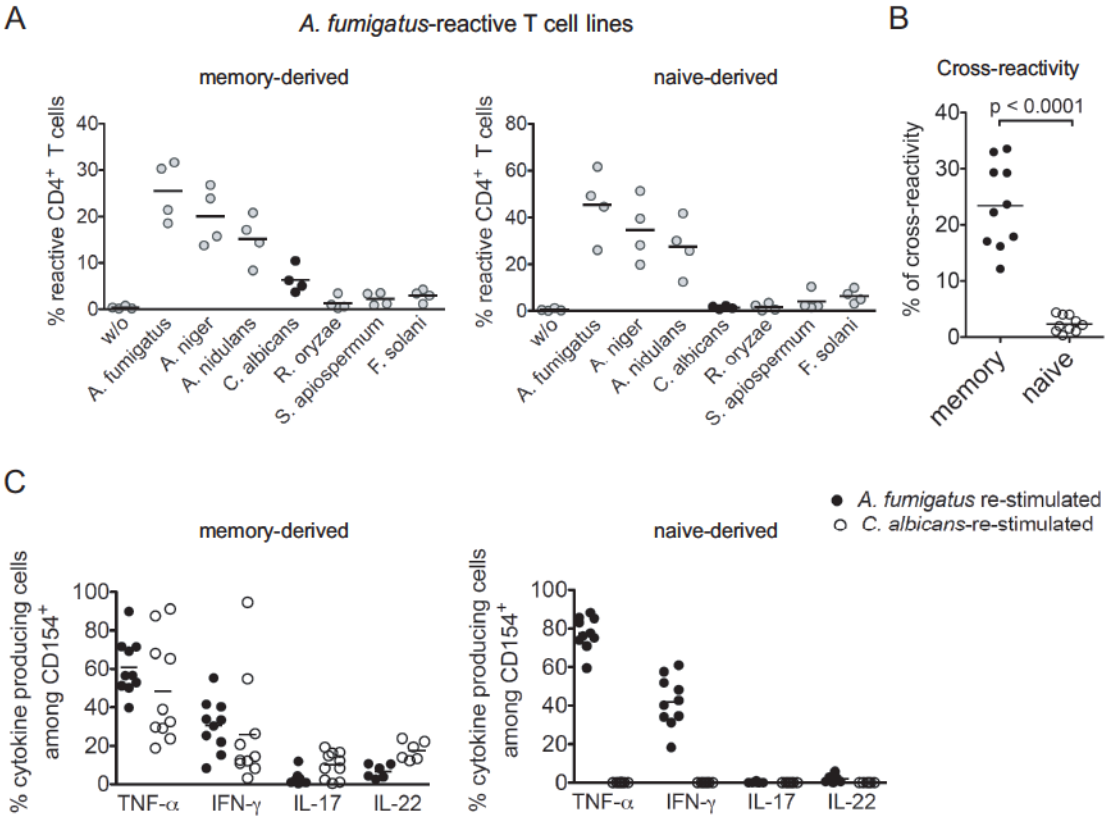
A
A. fumigatus-reactive T cell lines
memory-derived
naive-derived
% reactive CD4+ T cells
w/o
A. fumigatus
A. niger
A. nidulans
C. albicans
R. oryzae
S. apiospermum
F. solani
B
Cross-reactivity
p < 0.0001
% of cross-reactivity
memory
naive
C
A. fumigatus re-stimulated
C. albicans-re-stimulated
% cytokine producing cells among CD154+
TNF-α
IFN-γ
IL-17
IL-22

Figure 5

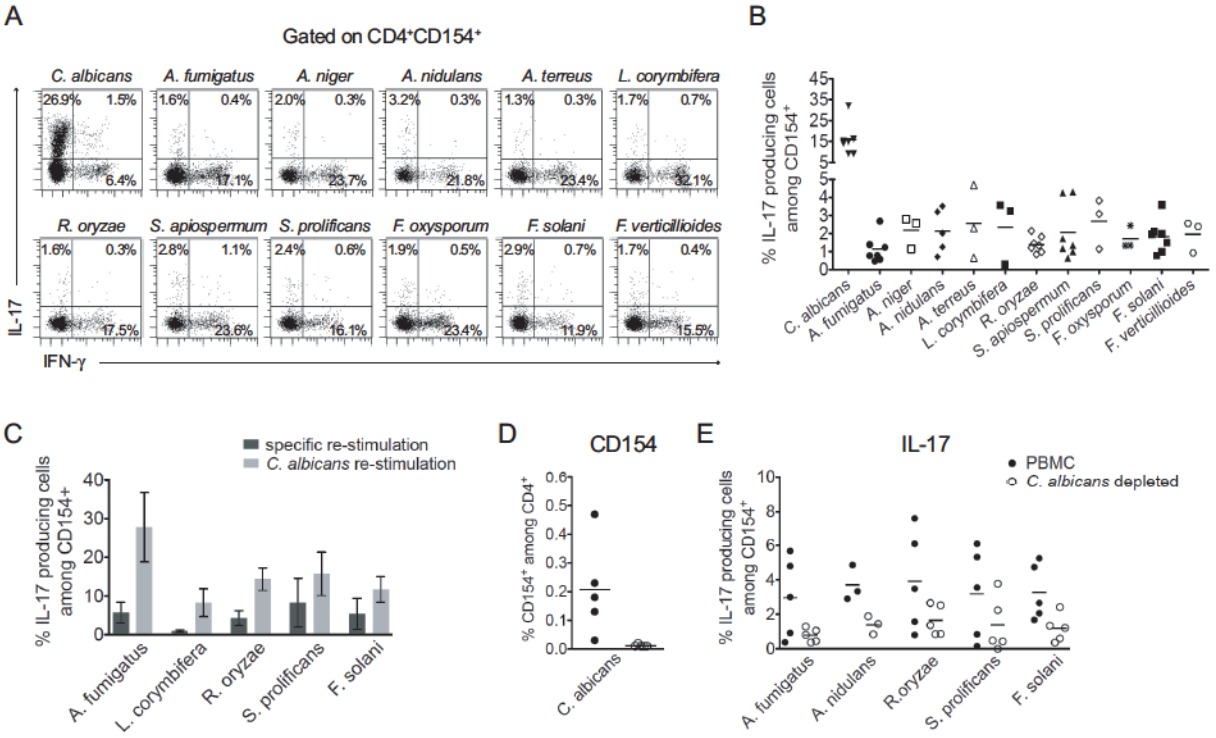
A
Gated on CD4+CD154+
C. albicans
A. fumigatus
A. niger
A. nidulans
A. terreus
L. corymbifera
R. oryzae
S. apiospermum
S. prolificans
F. oxysporum
F. solani
F. verticillioides
IL-17
IFN-γ
B
% IL-17 producing cells among CD154+
C
specific re-stimulation
C. albicans re-stimulation
% IL-17 producing cells among CD154+
D
CD154
% CD154+ among CD4+
E
IL-17
PBMC
C. albicans depleted
% IL-17 producing cells among CD154+

Figure S1

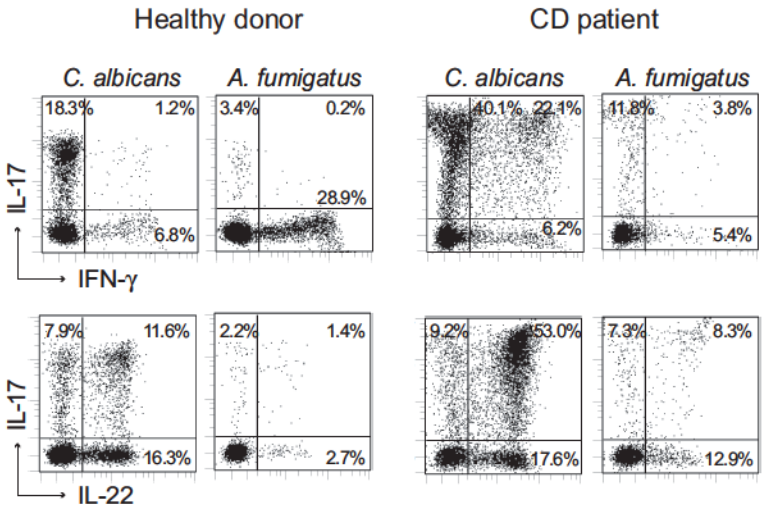

Figure S1. Qualitative modulation of fungus-specific T cell responses in Crohn´s disease patients. PBMCs of healthy donors or CD patients were stimulated with *C. albicans* or *A. fumigatus* lysate, reactive CD154$^+$ T cells were magnetically enriched and *ex vivo* analyzed for cytokine expression. Representative dot plot examples with percentage of cytokine producing cells among CD154$^+$ cells are shown.

Figure S2

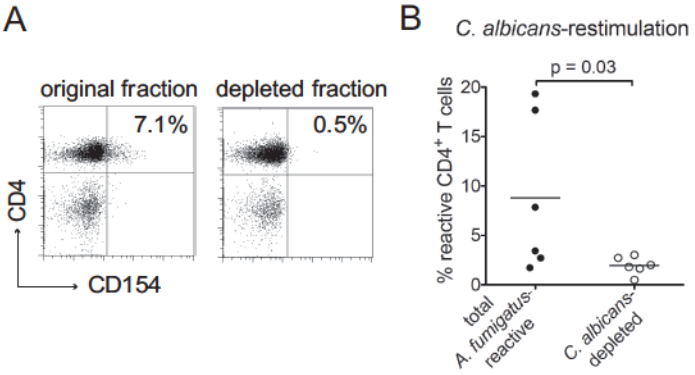

Figure S2. Efficient depletion of *C. albicans* cross-reactive cells from *A. fumigatus*-reactive T cell lines. *A. fumigatus*-reactive T cell lines generated via CD154-enrichment were re-stimulated with *C. albicans*-lysates and cross-reactive $CD154^+$ cells were depleted by MACS. (A) Dot plot examples for the original and depleted fraction with percentage of $CD154^+$ cells among $CD4^+$ and (B) statistical analysis for several donors are shown.

3.5 Manuscript V

"Rapid Diagnosis of Invasive Mold Infection by Measuring Fungus-Reactive $CD4^+$ T Cells in Peripheral Blood"

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* These authors contributed equally to this work.

Manuscript in preparation.

Rapid diagnosis of invasive mold infection by measuring fungus-reactive CD4$^+$ T cells in peripheral blood

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Short title: Fungus-reactive T cells in invasive mold infection

Key points:

1) $CD4^+$ T cells specific for clinically relevant fungi are present in all healthy individuals at relatively low and conserved frequencies.

2) Increased fungus-reactive T cell frequencies, but not the absolute number or effector cytokines, are indicative for patients with invasive fungal infections.

Abstract

Background

Invasive mold infections are difficult to recognize in immunocompromised patients, resulting in delayed and non-targeted therapy, which is a major cause for the extremely high mortality rates. Since $CD4^+$ T cells orchestrate anti-fungal immunity with high specificity, we analyzed whether quantification of fungus-specific T cells in peripheral blood may have diagnostic potential and also allows discrimination between the clinically most relevant fungal pathogens.

Methods

Following short *in vitro* stimulation with fungal lysates, reactive $CD154^+$ T cells were monitored by flow-cytometry in peripheral blood of healthy controls and patients at risk for, or with established diagnosis of invasive mold infection. Results were compared with the international consensus criteria for diagnosing invasive fungal infection.

Results

In 11 of 12 (91.7%) patients with proven or probable invasive fungal infection, mold-reactive $CD4^+$ lymphocytes were increased compared to healthy controls. In 24 of 26 (92.3%) patients at risk, but without established diagnosis of invasive fungal infection, mold-reactive $CD4^+$ lymphocyte frequencies were in the range of healthy donors ($P < .001$, odds ratio = 132 (95 % confidence interval, 10.79 to 1615.08)). Increased frequencies normalized during successful treatment. In contrast, effector cytokine production of mold-reactive T cells was variable and did not increase during infection. The genus-specificity of the T cell reaction allowed discriminating fungal pathogens and even identification of mixed infections with several fungi.

Conclusions

Measurement of fungus-reactive $CD154^+$ T helper cell frequencies in peripheral blood has the potential to identify invasive mold infection early and to allow targeted antifungal treatment.

Introduction

Ubiquitous molds cause life-threatening invasive infections in immunocompromised patients. The incidence of invasive mold infections is rising, and despite diagnostic and therapeutic improvements, mortality rates are striking. Leukemic patients and hematopoietic stem cell or solid organ transplant recipients are at particular risk.[1,2]

Early initiation of targeted antifungal therapy is the key to improved prognosis.[3,4] Still, fast and specific diagnostic methods and reliable markers are lacking.[5-7] Only the direct microscopic or cultural detection of the invading fungal pathogen in a sample taken from the infected site gives a clinical proof for the presence of an invasive fungal infection.[5] Such surgical procedures can rarely be applied to the majority of critically ill patients and rather represent a last therapeutic option, than a feasible diagnostic tool. The further classification of invasive fungal diagnosis into probable and possible cases requires a combination of the presence of a host factor (*e.g.* neutropenia), characteristic findings on lung computed tomography (CT) scans and microbiological findings[5] and thus relies on a high value of suspicion. As molds other than *Aspergillus* are emerging, also genus-specific discrimination becomes increasingly important.[8,9] However, even the histological examination of biopsy samples may not be specific enough to clearly distinguish similar filamentous fungal species.[23] Hence, there is an urgent need for improved diagnostic methods with high sensitivity and specificity; such methods should allow differentiating between fungal pathogens and enable immediate and targeted antifungal treatment.

Recent studies suggest that $CD4^+$ lymphocytes may contribute to host defense against fungi.[10-12] However, due to low frequencies and lack of knowledge about immune-dominant antigens, the actual frequency and phenotype of mold-reactive lymphocytes in healthy subjects and relevant patient groups remain elusive.

We previously showed, that the total pool of antigen-reactive T cells can be detected and characterized with high specificity and sensitivity based on the up-regulation of CD154 (CD40L) following brief *in vitro* stimulation of peripheral blood samples with fungal antigens.[13-15] Here, we used this sensitive technology to analyze mold-reactive $CD4^+$ T lymphocytes specific for predominate fungal species in peripheral blood of immunocompromised patients with or without fungal infection and healthy controls. We can show that mold-reactive T cell frequencies are significantly increased during invasive mold infection suggesting their usefulness as a highly specific though simple and robust read-out for genus-specific diagnosis of acute fungal infections from peripheral blood samples.

Methods

Peripheral blood sampling

Buffy coats from 100 healthy donors were obtained from the Institute for Transfusion Medicine, University Hospital Dortmund, Germany after informed consent. Peripheral ethylenediaminetetraacetic acid (EDTA) blood samples were collected from 45 patients at the University Hospital Cologne between July 2010 and August 2012. Blood sampling was performed within the scope of the biomaterial repository protocol ISI and all patients gave informed consent (local ethics identifier 08-160). The presence of at least two of the following criteria selected patients for participation: immunosuppression due to chemotherapy for underlying hematologic or oncologic malignancy, previous hematopoietic stem cell transplantation, prolonged immunosuppressive treatment, lung infiltrates on chest CT scan, positive galactomannan levels in serum (cut-off ≥ 0.5 in two consecutive samples) or bronchoalveolar lavage (BAL) fluid (cut-off ≥ 0.5 in a single sample), or clinical findings indicative of invasive mold infection. Patients donated at most 20 ml of peripheral blood twice weekly.

Preparation of fungal lysates

Each $2x10^6$ conidia/ml of *A. fumigatus* ATCC 46645 (LGC Standards, Wesel, Germany), *A. niger* FSU871, *A. nidulans* RMSO11, and *A. terreus* i402 were shaken for 20 hours with 200 rpm in YPD or AMM medium at 37 °C or 30 °C (for *A. niger*). Mycelia were recovered by filtration and disrupted in a Mikro-Dismembrator S (Sartorius Stedim Biotech GmbH, Göttingen, Germany) at 200 rpm for 10 min using 0.5 mm glass beads, as previously described.[15]

Clinical isolates of *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, *Lichtheimia corymbifera* (formerly *Absidia corymbifera*, *Mycocladus corymbifer), Rhizopus oryzae, Scedosporium apiospermum,* and *S. prolificans* obtained from the Fungiscope™ study were grown on Sabouraud dextrose agar plates until sporulation.[16] Conidia were harvested and shaken in RPMI-1640 medium supplemented with MOPS (3-(N-morpholino) propane sulfonic acid), glutamine and 2 % glucose for 5-7 days at 37 °C. Mycelia were disrupted using the gentleMACS™ device (Miltenyi Biotec, Bergisch Gladbach, Germany), program RNA.01 (5 times). All lysates were resuspended in 1x PBS buffer (2 mM $MgCl_2$), and centrifuged for 20 min at 20,000 g. Supernatants were stored in aliquots at -20° C until use.

Stimulation and enrichment of antigen-reactive T cells

Following Ficoll-Paque (GE Healthcare Life Sciences, Freiburg, Germany) density gradient centrifugation, peripheral blood mononuclear cells (PBMC) were resuspended in RPMI-1640 (Miltenyi Biotec), supplemented with 5% (v/v) human AB-serum (BioWhittaker/Lonza, Walkersville, MD, USA), 2 mM L-glutamine (PAA Laboratories, Pasching, Austria). Depending on the available amount, $5x10^5$ to $1x10^6$ PBMC were stimulated for 7 hours with 40 µg/ml of each fungal lysate or a pool thereof, or *Staphylococcus* enterotoxin B (SEB 1 µg/ml, Sigma-Aldrich, Schnelldorf, Germany), in presence of 1 µg/ml CD40, 1 µg/ml CD28 pure antibody (both Miltenyi Biotec).

For analysis of cytokine expression, $1x10^7$ PBMC were stimulated with the fungal lysates for 7 hours with 1 µg/ml Brefeldin A (Sigma Aldrich) added after 5 hours. $CD154^+$ cells were separated using the CD154 MicroBead Kit (Miltenyi Biotec). In brief, cells were indirectly magnetically labelled with CD154-Biotin and anti-Biotin microbeads and enriched by two sequential MS columns (Miltenyi Biotec), followed by intracellular cytokine staining (Inside stain Kit; Miltenyi Biotec).

Flow cytometry

Cells were stained with the following monoclonal antibodies according to manufacturer protocols: CD3-VioBlue (BW264/56), CD4-APC.Vio770, CD4-VioBlue (VIT4), CD8-PerCP (BW135/80), CD14-PerCP (TÜK4), CD20-PerCP (LT20), CD69-APC (FN50), CD154-PE, CD154-APC, CD154-VioBlue (5C8), TNF-α-FITC (cA2), IL10-PE (B-T10), IL17-APC (CZ8-23G1) (all Miltenyi Biotec), IFNγ-PerCP.Cy5.5 (4S.B3; BioLegend, San Diego, CA, USA). Data were acquired on a MACSQuant® analyzer and MACSQuantify™ software was used (Miltenyi Biotec).

Expansion and re-stimulation of antigen-specific T cells

Isolated $CD154^+$ cells were cultured at a density of 2.5×10^6 cells/cm^2 with 1:100 mitomycin C (Sigma Aldrich) treated autologous feeder cells in X-Vivo™15 (BioWhittaker/Lonza), supplemented with 5 % (v/v) AB-serum (BioWhittaker/Lonza), 200 U/ml IL-2 (Proleukin®; Novartis, Nürnberg, Germany) and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Antibiotic Antimycotic Solution, Sigma Aldrich). During expansion for 14 days, medium was replenished and cells were split as needed.

For re-stimulation, $5x10^5$ expanded T cells were combined 1:1 with autologous CD3-depleted PBMC and stimulated with the fungal lysates or CMV lysate (10 µg/ml, Siemens Healthcare Diagnostics, Marburg, Germany), and 1 µg/ml CD28 pure antibody for 6 hours; 1 µg/ml Brefeldin A (Sigma Aldrich) was added after 4 hours. Cells were stained for intracellular CD154 and cytokine expression (data not shown).

Clinical definitions

The clinical presence of invasive mold infection was diagnosed according to the 2008 consensus definitions of the European Organization for Research and Treatment of Cancer and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG).[5]

Statistical analysis

A cut-off value to discriminate between patients with and without invasive mold infection was determined by logistic regression analysis, followed by receiver operating characteristic (ROC) curve analysis using GraphPad PRISM® software 5.0.

Positive and negative T cell signals were compared to the clinical EORTC/MSG classification utilizing 2×2 contingency tables. Patients with proven and probable invasive mold infection were grouped *versus* those with possible or without such infection. Fisher's exact test was used to calculate the distribution of alignments between the current gold standard for invasive mold infection diagnosis and the T cell frequency quantification. A p-value of < 0.05 determined statistical significance.

Results

Mold-reactive $CD4^+$ lymphocyte frequencies in healthy donors

First we analyzed the frequency ranges of fungus-reactive $CD4^+$ T cells in healthy donors, following a 7-hour stimulation of PBMCs with various fungal lysates of the most frequent clinically isolated invasive fungal species. In healthy donors all fungal lysates induced a small, but clearly defined population of CD154 expressing $CD4^+$ T cells as compared to the non-stimulated control (Figure 1A). Analysis of different donors revealed that the highest frequencies of mold-reactive T cells were present against *A. fumigatus*, ranging from 0.1% to 0.3%, whereas frequencies against all other molds were below 0.15% in most donors (Figure 1B).

Patient characteristics

Next we analyzed patients at risk for invasive fungal infections. Of 45 patients analyzed, 6 had proven, 10 probable, 5 possible, and 24 patients had no invasive mold infection according to consensus criteria[5] (Table 1). The proportion of total $CD4^+$ cells ranged from 0.07% to 81% of PBMC (Supplemental Table 1), but flow-cytometric measurement of $CD154^+$ expression allowed for normalization of reactive T cell frequencies to the variable total $CD4^+$ T cell numbers. Since only low PBMC numbers could be obtained from immunocompromised patients, four different lysate pools (*Aspergillus* spp., *Fusarium* spp., Mucorales, *Scedosporium* spp.) were used for stimulation. Using pooled lysates did not increase the frequency ranges of fungus-reactive $CD4^+$ T cells in healthy donors (Supplemental Figure 1). We were able to determine the frequencies of mold-reactive T cells in 38 patients (84%; Supplemental Table 1). In five patients with T cell counts $< 0.15\%$ of PBMCs or with absence of antigen presenting cells, a CD154 expression was not inducible, and in two patients the background CD154 expression was too high.

Mold-reactive $CD4^+$ lymphocyte frequencies in patients with invasive mold infection

In a cohort of 100 healthy control donors, the mean proportions of *Aspergillus* spp.- and Mucorales-reactive $CD4^+$ lymphocytes were $0.14\% \pm 0.07\%$, and $0.06\% \pm 0.03\%$, respectively (Figure 2B). Compared to healthy donors some patients had markedly increased frequencies of mold-reactive $CD4^+$ cells (Figure 2A,B). Whenever possible, we monitored the frequencies of these patients at several time points. Despite some variability, mold-reactive $CD4^+$ T cell frequencies were generally higher than in healthy controls. After completion of

patient recruitment, we performed retrospective receiver-operating characteristic (ROC) with logistic regression analysis using normalized frequencies to determine the ideal cut-off value. Thus, an optimal cut-off value of 2.62 fold mean frequencies of healthy donors was calculated to separate patients with and without invasive mold infection (Figure 2C). The derived cut-offs were 0.37% for *Aspergillus* spp. and 0.15% for Mucorales, respectively.

Comparison of clinical diagnosis and mold-reactive $CD4^+$ lymphocyte frequencies

For comparison with the clinical diagnosis, patients with proven and probable invasive mold infection were grouped *versus* those with possible or without mold infection and contingency table analyses were performed. Using the derived cut-off values, diagnosis of invasive mold infection coincided in all 6 proven cases. When adjusting for patients with technically un-evaluable $CD4^+$ lymphocyte frequencies, 38 patients remained in the analysis. In 5 of 6 probable cases mold-reactive T cell frequencies were increased, and 24 of 26 patients with either possible or no invasive mold infection showed no increased frequencies, resulting in a sensitivity of 91.7% and a specificity of 92.3% (Supplemental Table 2). Positive and negative predictive values were 85% and 96%, respectively. The correlation between consensus diagnoses of invasive mold infection and mold-reactive $CD4^+$ lymphocyte frequencies was statistically significant (Fisher´s exact test, $P < .001$, odds ratio 132 (95% confidence interval 10.79 to 1615.08). Two patients had increased frequencies, but no diagnosis of invasive mold infection, thus formally representing false positive test results. One patient with a probable aspergillosis had no increased mold-reactive $CD4^+$ lymphocytes and was regarded as a false-negative result (Supplemental Table 2).

By including also patients with un-evaluable T cell frequencies, 5 of 10 probable cases had increased frequencies of mold-reactive $CD4^+$ lymphocytes and 27 of 29 cases with either possible or no invasive mold infection showed no increased frequencies, resulting in a sensitivity of 68.8% and a specificity of 93.1% (Supplemental Table 3). Again, the correlation between consensus diagnoses of invasive mold infection and mold-reactive $CD4^+$ lymphocyte frequencies was statistically significant (Fisher´s exact test, $P < .001$, odds ratio 29.7 (95% confidence interval 4.99 to 176.72). Possible invasive mold infection per definition lacks microbiological evidence. Analyzing our results excluding this ambiguous group did not lead to a relevant change of the results (Supplemental Table 4).

Mold-specific T cells during the course of disease

We also monitored patients with elevated frequencies of fungi-reactive T cells at several time points. Interestingly, whereas elevated T cell frequencies remained high during the course of proven invasive mold infection, elevated frequencies of Mucorales-reactive $CD4^+$ T cells rapidly dropped in two patients after removal of the infected tissue by lung resection (Figure 3, Supplemental Table 1). This indicated that the increased frequencies represent a specific marker for acute fungal infections.

Discrimination between invading fungal pathogens

Next we analyzed, whether the measurement of fungus-reactive T cell frequencies allows discrimination between different fungal pathogens. In three patients with histologically proven aspergillosis, T cell frequencies against *Aspergillus* spp. pooled lysate were increased. Also, in two patients with histologically proven mucormycosis infection elevated T cell frequencies against Mucorales pooled lysate were detected (Figure 2B, Table 1). One patient with histologically proven aspergillosis showed elevated proportions of Mucorales-reactive T cells. One other patient with proven invasive aspergillosis showed an increase in T cell frequencies to Mucorales, to *Scedosporium* spp., and to *Fusarium* spp. pooled lysate in addition to increased *Aspergillus* spp.-reactive T cells (Supplemental Table 1), indicating potential of the T cell assay to identify pathogenic fungi even in mixed infection.

However, for a species-specific discrimination, T cell cross-reactivity against different fungal species has to be taken into account. Fungus-specific T cell lines were generated from healthy donors and their specificity was tested via antigen re-stimulation. Expanded mold-specific T cell lines were highly reactive (40-80 %) against the fungal lysate used for the initial stimulation, as shown by re-expression of CD154 and TNF-α (Figure 4). Reactivity against CMV lysate as a control antigen was not above the background level. *A. fumigatus*-reactive T cell lines cross-reacted against *A. nidulans* and vice versa, as well as other *Aspergillus* species (data not shown), indicating that discrimination on species level may be difficult. In contrast, the T cell cross-reactivity of the different fungus-specific T cell lines against more distantly related fungal species was low. However, *S. apiospermum*- and to a lower extent *F. solani*-reactive T cells also responded upon stimulation with other fungal species. Importantly, we observed only low cross-reactivity between *Aspergillus* spp.-reactive T cells and the Mucorales species *Rhizopus oryzae.*

Effector cytokine production was not increased during fungal infection

The cytokine expression of fungus-reactive T cells was analyzed in healthy controls and in patients with or without increased frequencies of *Aspergillus*-spp.-reactive T cell responses. Following magnetic pre-enrichment and multi-parametric flow-cytometric analysis of CD154 expressing $CD4^+$ T cells, the contribution of TNF-α, IFN-γ, IL-10 and IL-17 expressing T cells to the total fungi-specific $CD4^+$ T cell pool was analyzed by intracellular cytokine staining. Independently of the fungal species used for stimulation, cytokines were produced only by small and highly variable proportion of all antigen-activated $CD154^+$ T cells in healthy donors (Figure 5A, B). Interestingly, very similar frequencies of cytokine producers were found in patients irrespective of the infection status, although patients with proven/probable infection, *i.e.* increased frequencies of *Aspergillus*-spp.-reactive T cells, tended to have rather high frequencies of TNF-α producers (Figure 5C, D). However, the overlapping frequencies of cytokine producing T cells in patients with and without invasive fungal infection indicated the limited use of cytokine production as a diagnostic tool in comparison to the quantitation of the total reactive T cells frequencies.

Discussion

In this study, we analyzed the mold-reactive proportion among $CD4^+$ lymphocytes in peripheral blood from immunocompromised hosts and healthy controls using a sensitive flow-cytometric detection assay. We found a significant correlation of mold-reactive $CD4^+$ T cell frequencies with invasive mold infection.

Healthy donors presented mold-reactive T cells at very low but conserved frequency ranges, likely reflecting chronic exposure to ubiquitous fungi. In patients with invasive mold infection, increased frequencies of mold-reactive T cells allowed to determine a cut-off value. Across various types of immunocompromised patients, the test yielded a sensitivity of 68.8% and a specificity of 93.1%. When applied to patients without severe T cell deficiency, sensitivity increased to 91.7% and specificity remained as high as 92.3%. Of note, there was only one false negative test result when excluding the easily discernible patients with insufficient T cell numbers. The lack of an increased T cell signal in this single patient might be due to our panel of lysates not covering all potential fungal pathogens. Alternatively, since the patient only had a probable infection based on surrogate markers, this patient may indeed not have had a fungal infection. Two immunocompromised patients displaying increased T cell signals did not show clinical signs or symptoms of invasive mold infection. Since we detected no such increase in the healthy control group, it is also conceivable that these patients had undiagnosed fungal infection. However, considering the single probable case formally as false negative and the two patients with no IMI as false positive, the measurement of fungus-reactive T cells still resulted in a high sensitivity and specificity exceeding results of currently available blood tests for invasive mold infections, i.e. galactomannan, PCR and 1,3-β-D-glucan.[6,17,18]

Cytokine ELISpot assay has been proposed as a diagnostic tool for fungal infections.[19,20] However, we observed that cytokine producers represent a rather small and highly variable subset within the total fungus-reactive T cell pool already in healthy donors, as well as patients with or without fungal infection, questioning the significance and reliability of cytokine production as a diagnostic parameter. In addition, increased frequencies of mold-reactive T cells could only be identified upon normalization to the highly variable T cell counts found in these patients, which can not easily be accomplished for ELISpot or ELISA data. Interestingly, the production of important effector cytokines, such as IFN-γ or IL-17, which have been associated with protection from fungal infection, were not increased but even rather low compared to healthy controls. This suggests that expansion of mold-reactive

T cells during invasive infection is not accompanied by acquisition of full effector functions. In addition, only the relative frequencies but not the absolute numbers of mold-reactive $CD4^+$ T cells were increased in infected patients (Supplemental Figure 2). This may explain why invasive mold infections are not effectively cleared despite the infection-related expansion of fungus-specific T helper cells.

While the galactomannan enzyme immunoassay is an accepted standard, it is restricted to aspergillosis.[5] However, in aspergillosis, galactomannan slope has been a helpful prognostic marker.[21] It is particularly interesting that in patients with invasive mold infection the slope of the specific $CD4^+$ lymphocyte frequencies associated with the burden of fungal disease, *i.e.* decreased after surgical resection. These data emphasize the measurement of specific $CD4^+$ lymphocyte frequencies as a reliable and non-invasive test for mold infections. Since early treatment improves outcome,[22] another encouraging observation is that the fungus-reactive T cell detection may precede the clinical diagnosis of invasive mold infected patients by weeks (data not shown).

Discrimination between causative fungal species poses a challenge in the diagnosis of invasive mold infection. Certain filamentous fungi are difficult to distinguish even in histology.[23] In one individual with proven aspergillosis, Mucorales-reactive T cell frequencies were increased whereas frequencies of *Aspergillus* spp.-reactive T cells were not. Histology of lung tissue was indicative of double infection and moreover, the frequencies of Mucorales-reactive T cells rapidly decreased following lung resection, further arguing for an infection with Mucorales. To characterize the species specificity of the T cell reaction, cross-reactivity of expanded T cell lines was analyzed. Re-stimulation revealed only limited cross-reactivity against distantly related fungal pathogens, indicating that measurement of fungal T cell reactions allows clear discrimination between *Aspergillus* spp.- and Mucorales-reactive T cell responses. We observed substantial cross-reactivity between different *Aspergillus* strains, as well as between *Aspergillus* spp., *S. apiospermum* and *F. solani*, indicating that discrimination on the species level may require more detailed analysis. However, this surprisingly high level of T cell cross-reactivity against certain fungal species may have important implications for the design of immunotherapies or vaccinations.

The assay described here, is restricted to patients with an *in vitro* inducible T cell response. Of the population analyzed, 16% were un-evaluable because of either very low T cell counts (a limitation which may be overcome by larger blood samples) or the absence of antigen presenting cells, or a pronounced background CD154 expression. Recent treatment with

alemtuzumab or purine analogues or advanced HIV infection might interfere with our test by depleting T cells.

In summary, we provide a simple peripheral blood assay to analyse fungus-reactive T cells in healthy donors as well as in patient groups at risk for fungal infections. Mold-reactive T cells are present in all donors at low but conserved frequency ranges. Increased frequencies identified invasive mold infection with high sensitivity and specificity whereas cytokine production seems not to be significantly altered. The slope of increased frequencies was associated with invasive fungal disease burden. The assay has the potential to discriminate between pathogenic fungi and to identify mixed fungal infections to enable immediate and targeted antifungal treatment.

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Authorship contributions

P.B. performed experiments; P.B., A.Sc. designed experiments; A.St., M.J.G.T.V., J.J.V., O.A.C. recruited study participants; O.K., A.H., A.A.B. generated fungal extracts; P.B., A.St., O.K., A.H., M.A., M.J.G.T.V., J.J.V., A.A.B., O.A.C., A.Sc. interpreted results and wrote the manuscript.

Conflict of interest disclosure

M.A. is employee of Miltenyi Biotec. MJGTV has received research grants from 3M and lecture honoraria from Pfizer, Gilead, Astellas and Merck. JJV has received research grants from Astellas, Infectopharm, Pfizer and Merck/Schering, and has served on the speakers´ bureau of Merck/Schering, and Pfizer. OAC has received research grants from 3M, Actelion, Astellas, Basilea, Bayer, Biocryst, Celgene, Cubist, F2G, Genzyme, Gilead, GSK, Merck/Schering, Miltenyi, Optimer, Pfizer, Quintiles, and Viropharma, is a consultant to 3M, Astellas, Basilea, Cubist, F2G, Gilead, GSK, Merck/Schering, Optimer, Pfizer and Sanofi Pasteur, and received lecture honoraria from Astellas, Gilead, Merck/Schering, and Pfizer. A.S. works as a consultant for Miltenyi Biotec. All other authors declare no competing financial interests.

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Figure legends

Figure 1. Fungus-reactive T cell frequencies among peripheral blood $CD4^+$ T cells in healthy volunteers. PBMCs of healthy donors were stimulated for 7 hours with the indicated fungal lysates and analysed for $CD154^+$ expression. To optimize the detection and quantification of $CD154^+$ events among $CD3^+CD4^+$ T cells, cell aggregates (scatter area versus scatter height), dead cells and non-T cell lineages ($CD14^+$, $CD20^+$, dump) were excluded, and cells were counterstained for the activation marker CD69. (A) Representative dot plot examples with frequencies of $CD154^+CD69^+$ cells among $CD4^+$ T lymphocytes. (B) Statistical analysis of several donors with horizontal lines indicating mean values (n=18).

Figure 2. Frequencies of fungus-reactive T cells in patients with invasive mold infections. (A) Representative dot plot examples of two patients with increased frequencies of *Aspergillus* spp.- and Mucorales-reactive T cells, respectively. Percentages of $CD4^+$ T cells in PBMC and $CD154^+CD69^+$ cells among $CD4^+$ lymphocytes are indicated. (B) Frequencies of *Aspergillus* spp.- and Mucorales-reactive T cells in PBMCs of healthy control subjects (black dots; n=100) and high-risk patients without an invasive mold infection (black boxes; n=24) and with a proven or probable invasive mold infection according to EORTC/MSG consensus criteria. Patients with increased reactive T cell measurements for *Aspergillus* spp. are depicted as red/orange symbols (n=6, in total 38 patients analyzed) and/or for Mucorales as dark/light blue symbols (n=6, in total 23 patients analyzed). (C) An optimized cut-off value to discriminate between patients with and without invasive mold infection was calculated by linear regression and receiver operating characteristic (ROC) curve analysis and normalized to the mean value of healthy control subjects (sensitivity 91.7%, specificity 92.3%).

Figure 3. Fungus-reactive T cell frequencies in relation to burden of fungal disease. Fungus-reactive T cells of two patients with increased Mucorales-reactive T cell frequencies were monitored over several weeks. Dashed lines represent the cut-off of 0.15% Mucorales-reactive T cells discriminating patients with and without invasive mold infection.

Figure 4. Re-stimulation of expanded fungus-reactive T cell lines. Fungus-specific T cell lines were generated from healthy donors and tested for specificity via antigen re-stimulation. (A) Representative dot plot examples of one donor with percentage of reactive cells among

$CD4^+$ lymphocytes are shown. (B) Statistical analysis of several donors with horizontal lines indicating mean values (n=7).

Figure 5. Cytokine expression of fungus-reactive $CD4^+$ T cells. Following stimulation with fungal lysates, reactive $CD154^+$ cells were magnetically enriched and stained intracellular for cytokine expression. (A) Representative dot plot examples with percentages of cytokine producing cells among $CD154^+$ of one healthy donor or (B) statistical analysis of several healthy donors. (C) Cytokine expression of *Aspergillus* spp.-reactive T cells in two patients with increased *Aspergillus* spp-reactive T cell frequencies and (D) statistical analysis of several patients with or without fungal infection (in part several measurements were performed from the same patient.)

Table 1. Classification of Invasive Mold Infection According to the 2008 EORTC/MSG Criteria, and Mold-Reactive CD4$^+$ Lymphocyte Frequencies

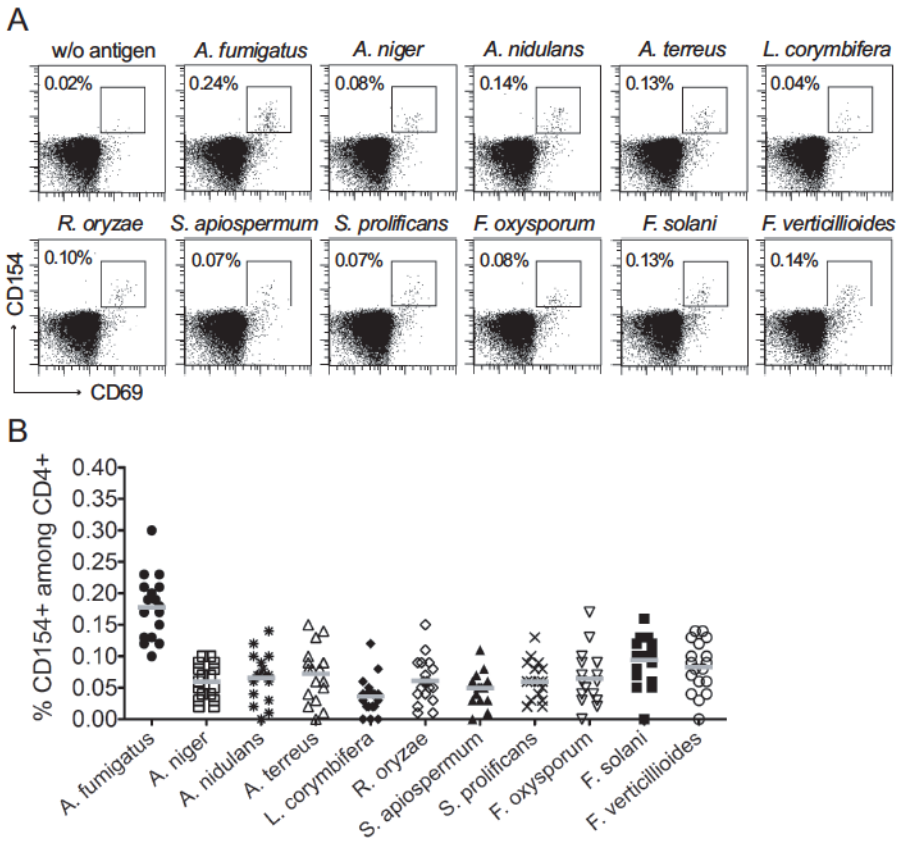
Patient	Underlying Disease	Host Risk Factor	Chest Computed Tomography Indicative of Invasive Mold Infection	Galactomannan Index*	Histology	Fungal Culture	Polymerase chain reaction	Level of Diagnosis of Invasive Mold Infection[5]	Mold for which Elevated T Cell Frequencies were Detected
1	Hodgkin's lymphoma	No known risk factor	Yes	Serum pos. BAL ND	*Aspergillus spp.*[†]	ND	ND	Proven	*Aspergillus* spp.
2	Acute myeloid leukemia	Neutropenia	Yes	Serum neg. BAL pos.	*Aspergillus spp.*[†,‡]	ND	*A. fumigatus*[‡]	Proven	*Aspergillus* spp.
3	Acute myeloid leukemia	Neutropenia	Yes	BAL neg. Serum neg.	*Aspergillus spp.*[‡]	No growth[‡]	No fungal evidence[‡,§]	Proven	Mucorales
4	Multiple myeloma	No known risk factor	Yes	BAL neg. Serum neg.	Mucorales[‡]	No growth[†,‡,§]	*Aspergillus spp.*[§] *Rhizopus microsporus*[§]	Proven	Mucorales
5	Acute myeloid leukemia	Neutropenia	Yes	BAL neg. Serum neg.	Mucorales[‡]	No growth[‡,§]	*Rhizopus spp.*[‡]	Proven	Mucorales
6	Rheumatoid arthritis	Glucocorticosteroids	Yes	BAL pos. Serum neg.	*Aspergillus spp.*[‡]	*A. fumigatus* complex[‡]	*Fusarium merismoides*[†] *A. fumigatus* complex[‡]	Proven	*Aspergillus* spp., Mucorales *Scedosporium* spp., *Fusarium* spp.
7	Acute promyelocytic leukemia	Neutropenia	Yes	BAL pos. Serum neg.	No fungus[†,§]	No growth[†]	*Rhizopus spp.*[†]	Probable	Mucorales
8	Chronic lymphocytic leukemia	Neutropenia	Yes	BAL pos. Serum neg.	NA	No growth[§]	No fungal evidence[§]	Probable	Mucorales
9	Mantle cell lymphoma	Neutropenia	Yes	BAL pos. Serum pos.	NA	No fungal growth[§]	ND	Probable	*Aspergillus* spp.
10	Non-Hodgkin's lymphoma	Neutropenia[¤]	Yes	BAL pos. Serum neg.	NA	No fungal growth[§]	ND	Probable	*Aspergillus* spp.
11	Acute lymphoblastic leukemia	Neutropenia	Yes	BAL pos. Serum pos.	No fungus[†]	No growth[†,§]	*Aspergillus spp.*[†]	Probable	*Aspergillus* spp.
12	Acute lymphoblastic leukemia	Neutropenia	Yes	BAL pos.	NA	No fungal growth[§]	ND	Probable	None
13	Chronic lymphocytic leukemia	Neutropenia	Yes	BAL pos. Serum pos.	NA	*Aspergillus flavus*[§]	ND	Probable	No induction of CD154 expression due to nearly undetectable numbers of T cells

14	Aplastic anemia	Neutropenia	Yes	BAL neg. Serum pos.	No fungus†	No growth§	*Aspergillus spp.*§ No fungus†	Probable	CD154 background expression too high to determine fungi-reactive T cell frequencies
15	Multiple myeloma	Neutropenia	Yes	BAL ND Serum pos.	NA	NA	NA	Probable	CD154 background expression too high to determine fungi-reactive T cell frequencies
16	Acute myeloid leukemia	Neutropenia	Yes	BAL pos. Serum neg.	NA	ND	ND	Probable	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells
17	Acute myeloid leukemia	Neutropenia	Yes	BAL neg. Serum pos.	NA	No fungal growth§	ND	Possible	None
18	Acute lymphoblastic leukemia	Neutropenia	Yes	BAL neg. Serum ND	NA	No fungal growth§	ND	Possible	None
19	Acute myeloid leukemia	Neutropenia	Yes	BAL neg. Serum neg.	NA	No fungal growth§	ND	Possible	None
20	Acute lymphoblastic leukemia	Neutropenia	Yes	BAL neg. Serum neg.	NA	No fungal growth§	ND	Possible	None
21	Acute myeloid leukemia	Neutropenia	Yes	BAL neg. Serum neg.	NA	No fungal growth§	ND	Possible	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells
22	T cell leukemia	Neutropenia	No	BAL ND Serum neg.	NA	NA	NA	No	None
23	Acute myeloid leukemia	Neutropenia	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	None
24	Acute myeloid leukemia	Neutropenia	No	BAL neg. Serum neg.	NA	ND	ND	No	None
25	Acute myeloid leukemia	Neutropenia	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	None
26	Allogeneic HSCT	Allogeneic HSCT	No	BAL ND Serum neg.	NA	NA	NA	No	None
27	Allogeneic HSCT	Allogeneic HSCT	No	BAL ND Serum ND	NA	NA	NA	No	None
28	Non-Hodgkin's lymphoma	None	Yes	BAL ND Serum neg.	NA	NA	NA	No	None
29	Acute T cell leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
30	Acute lymphoblastic leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None

31	Acute myeloid leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
32	Acute myeloid leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
33	Acute T cell leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
34	Acute lymphoblastic leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
35	Acute myeloid leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
36	Acute lymphoblastic leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
37	Acute lymphoblastic leukemia	None	ND	BAL ND Serum ND	NA	NA	NA	No	None
38	Hodgkin lymphoma	None	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	None
39	Allogeneic HSCT	Allogeneic HSCT	No	BAL neg. Serum ND	NA	No fungal growth§	ND	No	None
40	CUP-syndrome	None	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	None
41	Acute myeloid leukemia	None	No	BAL ND Serum ND	NA	NA	NA	No	None
42	Acute myeloid leukemia	Neutropenia	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	*Aspergillus* spp.
43	Acute myeloid leukemia	None	Yes	BAL neg. Serum neg.	NA	No fungal growth§	*Aspergillus spp.*§	No	Mucorales *Scedosporium* spp.
44	Non-Hodgkin's lymphoma	None	No	BAL neg. Serum ND	NA	No fungal growth§	ND	No	No induction of CD154 expression due to nearly undetectable numbers of T cells
45	Hodgkin's lymphoma	None	No	BAL neg. Serum neg.	NA	No fungal growth§	ND	No	No induction of CD154 expression due to nearly undetectable numbers of T cells

* Cut-off to positivity ≥ 0.5 in two consecutive serum samples, OR ≥ 0.5 in one BAL sample;
† Lung biopsy;
‡ Lung resection;
§ Bronchoalveolar lavage fluid;
⍰ Only 8 days of neutropenia;
BAL, bronchoalveolar lavage; ND, not done; NA, not applicable; HSCT, hematopoietic stem cell transplantation; CUP, cancer of unknown primary.

Figure 1


A
w/o antigen
A. fumigatus
A. niger
A. nidulans
A. terreus
L. corymbifera
0.02%
0.24%
0.08%
0.14%
0.13%
0.04%
R. oryzae
S. apiospermum
S. prolificans
F. oxysporum
F. solani
F. verticillioides
0.10%
0.07%
0.07%
0.08%
0.13%
0.14%
CD154
CD69
B
% CD154+ among CD4+
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
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A. fumigatus
A. niger
A. nidulans
A. terreus
L. corymbifera
R. oryzae
S. apiospermum
S. prolificans
F. oxysporum
F. solani
F. verticillioides

Figure 2

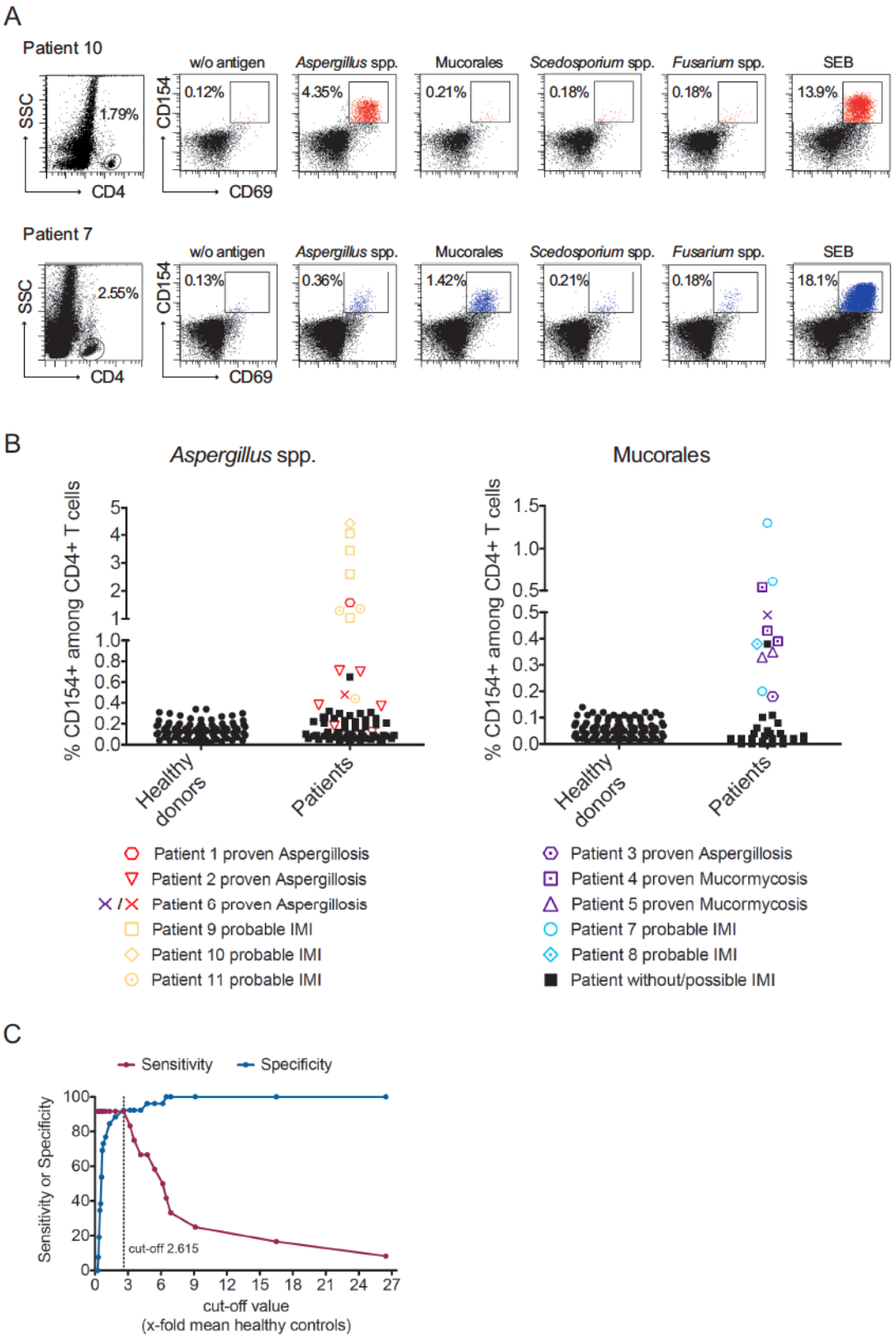

A
Patient 10
w/o antigen
Aspergillus spp.
Mucorales
Scedosporium spp.
Fusarium spp.
SEB
SSC
CD4
CD154
CD69
1.79%
0.12%
4.35%
0.21%
0.18%
0.18%
13.9%
Patient 7
2.55%
0.13%
0.36%
1.42%
0.21%
0.18%
18.1%
B
Aspergillus spp.
Mucorales
% CD154+ among CD4+ T cells
Healthy donors
Patients
Patient 1 proven Aspergillosis
Patient 2 proven Aspergillosis
Patient 6 proven Aspergillosis
Patient 9 probable IMI
Patient 10 probable IMI
Patient 11 probable IMI
Patient 3 proven Aspergillosis
Patient 4 proven Mucormycosis
Patient 5 proven Mucormycosis
Patient 7 probable IMI
Patient 8 probable IMI
Patient without/possible IMI
C
Sensitivity
Specificity
Sensitivity or Specificity
cut-off 2.615
cut-off value
(x-fold mean healthy controls)

Figure 3

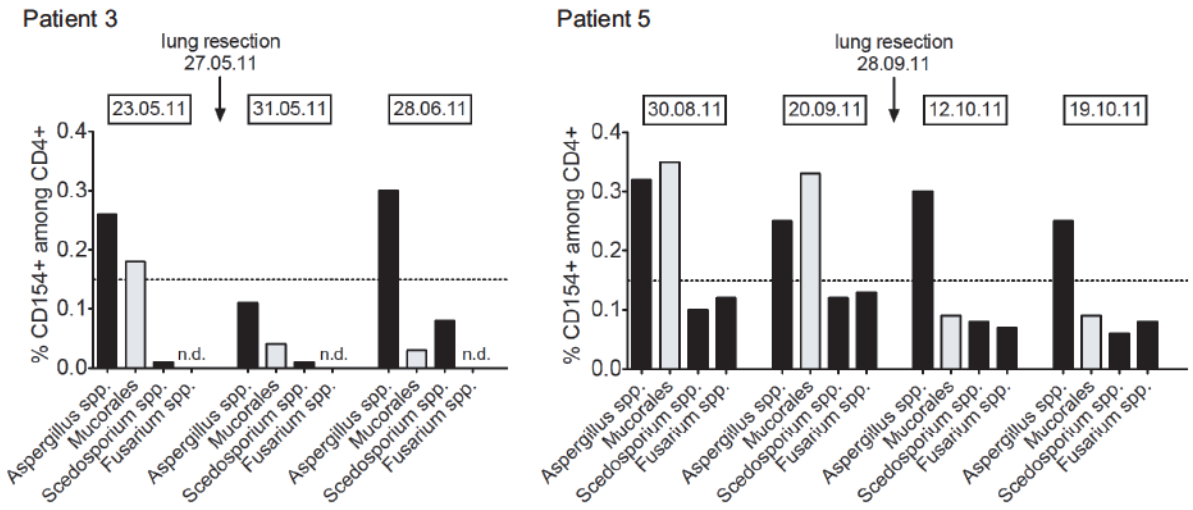

Patient 3
lung resection
27.05.11
23.05.11
31.05.11
28.06.11
% CD154+ among CD4+
0.4
0.3
0.2
0.1
0.0
n.d.
n.d.
n.d.
Aspergillus spp.
Mucorales
Scedosporium spp.
Fusarium spp.
Patient 5
lung resection
28.09.11
30.08.11
20.09.11
12.10.11
19.10.11

Figure 4

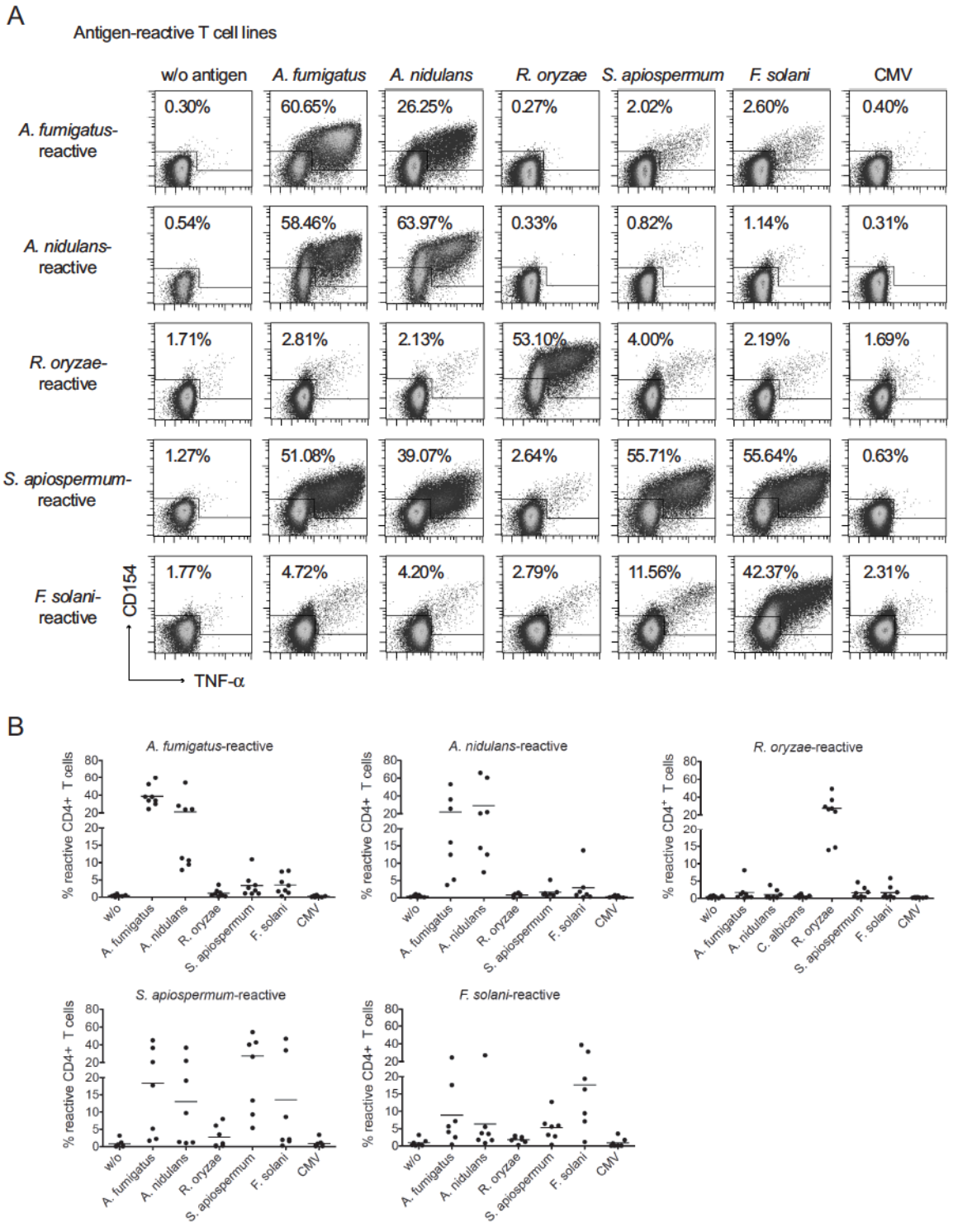

A
Antigen-reactive T cell lines
w/o antigen
A. fumigatus
A. nidulans
R. oryzae
S. apiospermum
F. solani
CMV
A. fumigatus-reactive
A. nidulans-reactive
R. oryzae-reactive
S. apiospermum-reactive
F. solani-reactive
0.30%
60.65%
26.25%
0.27%
2.02%
2.60%
0.40%
0.54%
58.46%
63.97%
0.33%
0.82%
1.14%
0.31%
1.71%
2.81%
2.13%
53.10%
4.00%
2.19%
1.69%
1.27%
51.08%
39.07%
2.64%
55.71%
55.64%
0.63%
1.77%
4.72%
4.20%
2.79%
11.56%
42.37%
2.31%
CD154
TNF-α
B
A. fumigatus-reactive
A. nidulans-reactive
R. oryzae-reactive
S. apiospermum-reactive
F. solani-reactive
% reactive CD4+ T cells
w/o
A. fumigatus
A. nidulans
C. albicans
R. oryzae
S. apiospermum
F. solani
CMV

Figure 5

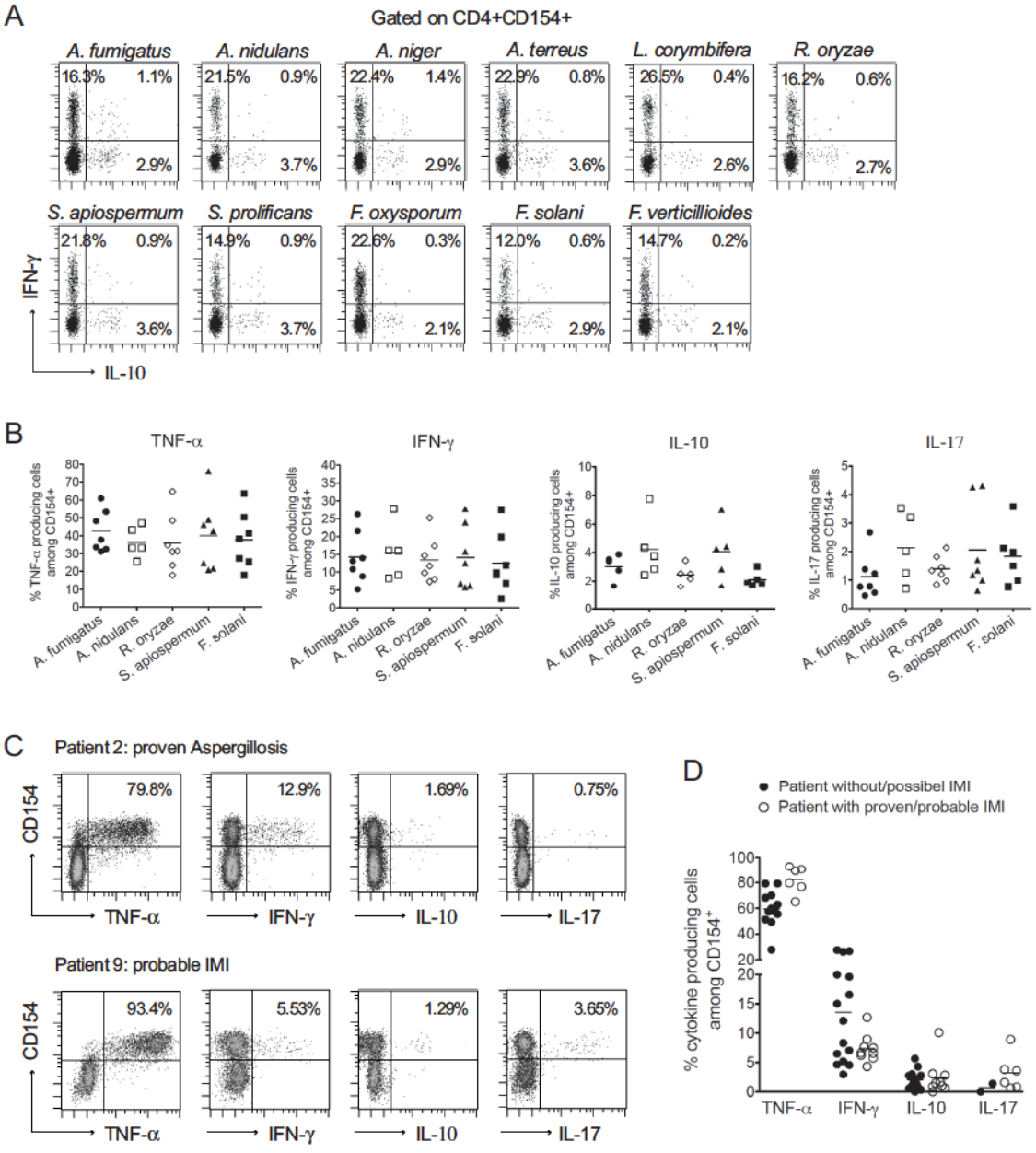

A
Gated on CD4+CD154+
A. fumigatus
A. nidulans
A. niger
A. terreus
L. corymbifera
R. oryzae
S. apiospermum
S. prolificans
F. oxysporum
F. solani
F. verticillioides
IFN-γ
IL-10
B
TNF-α
IFN-γ
IL-10
IL-17
% TNF-α producing cells among CD154+
% IFN-γ producing cells among CD154+
% IL-10 producing cells among CD154+
% IL-17 producing cells among CD154+
A. fumigatus
A. nidulans
R. oryzae
S. apiospermum
F. solani
C
Patient 2: proven Aspergillosis
CD154
TNF-α
IFN-γ
IL-10
IL-17
Patient 9: probable IMI
D
Patient without/possibel IMI
Patient with proven/probable IMI
% cytokine producing cells among CD154+

Table S1. Classification of Invasive Mold Infection According to the 2008 EORTC/MSG Criteria, Histological Evidence and Quantification of Mold-Reactive $CD4^+$ Lymphocyte Frequencies

Patient	Underlying Disease	Histology			Level of Diagnosis of Invasive Mold Infection[5]	Date of Blood Sampling	WBC Count [x1E9/l]*	Lymphocyte Count [x1E9/l]*	$CD4^+$ [%] in PBMC	T cell Frequency			
		Date of Sampling	Sample Type	Result						*Aspergillus* spp.	Mucorales	*Scedoporium* spp.	*Fusarium* spp.
1	Hodgkin's lymphoma	08.11.10	Lung biopsy	*Aspergillus spp.*	Proven	08.16.10	4.29	0.39	1.44	1.58	-†	-†	-†
2	Acute myeloid leukemia	07.16.10	Lung biopsy	*Aspergillus spp.*	Proven	07.27.10	17.65	1.89	15.58	0.71	-†	-†	-†
						08.04.10	8.08	1.39	23.34	0.38	-†	-†	-†
						08.11.10	4.98	1.09	38.34	0.18	-†	-†	-†
		10.13.10	Lung resection	*Aspergillus spp.*		08.23.10	0.22	1.12	39.10	0.12	-†	-†	-†
						08.30.10	3.66	1.32	24.50	0.70	-†	-†	-†
						09.09.10	3.36	1.02	24.50	0.37	-†	-†	-†
3	Acute myeloid leukemia	05.27.11	Lung resection	*Aspergillus spp.*, suspected Mucorales	Proven	05.17.11	3.31	0.92	3.80	0.05	-†	-†	-†
						05.23.11	4.72	0.86	4.80	0.26	0.18	0.01	-†
						05.31.11	3.43	ND	10.50	0.11	0.04	0.01	-†
						06.28.11	0.16	0.02	2.96	0.03	0.03	-‡	-‡
4	Multiple myeloma	08.08.11	Lung biopsy	No fungal evidence	Proven	08.11.11	8.95	0.81	16.50	0.12	0.39	0.06	0
		08.26.11	Lung biopsy	No fungal evidence		08.18.11	ND	ND	7.23	0.11	0.43	0.04	0.04
		09.21.11	Lung resection	Mucorales		08.26.11	5.17	0.41	9.24	0.24	0.54	0.13	0.12
5	Acute myeloid leukemia	09.29.11	Lung resection	Mucorales	Proven	08.30.11	6.62	0.35	4.33	0.32	0.35	0.1	0.12
						09.20.11	6.48	1.00	2.44	0.25	0.33	0.12	0.13
						10.12.11	1.71	1.28	11.91	0.30	0.09	0.08	0.07
						10.19.11	1.32	0.84	10.90	0.25	0.09	0.06	0.08
6	Rheumatoid arthritis	02.21.12	Lung biopsy	Interstitial lung disease	Proven	02.09.12	2.72	0.38	31.47	0.06	0.05	0.04	0.02
		03.13.12	Lung resection	*Aspergillus spp.*		03.15.12	18.15	0.65	1.38	0.48	0.49	0.52	0.57

7	Acute promyelocytic leukemia	02.17.12	Lung biopsy	No fungal evidence	Probable	01.12.12	0.43	ND	45.68	0.04	0.04	0.04	0.09
						01.17.12	0.32	0.27	29.51	0.04	0.02	0.02	0.02
						02.07.12	0.96	0.12	4.30	0.05	0.04	0.03	0.04
						02.28.12	10.50	1.30	2.55	0.23	1.29	0.08	0.05
						04.05.12	9.69	2.61	9.08	0.23	0.61	0.06	0.09
						04.19.12	10.00	2.18	7.35	0.15	0.2	0.04	0.05
						04.24.12	4.21	1.43	6.56	0.13	0.12	0.03	0.09
8	Chronic lymphocytic leukemia	ND	NA	NA	Probable	02.08.11	19.91	ANP	0.35	0.10	0.03	0.06	-†
						02.16.11	4.64	4.35	0.86	0.30	0.38	0.06	-†
9	Mantle cell lymphoma	ND	NA	NA	Probable	05.17.11	12.37	0.19	1.26	1.03	-†	-†	-†
						05.23.11	15.45	1.44	0.32	4.05	-†	-†	-†
						05.31.11	7.71	0.77	5.31	2.60	-†	-†	-†
						06.06.11	10.09	ND	2.76	3.44	-†	-†	-†
10	Non-Hodgkin's lymphoma	ND	NA	NA	Probable	01.13.12	10.73	0.73	1.87	4.32	0.09	0.06	0.06
						01.18.12	7.49	0.32	7.61	0.02	0.08	0.05	0.02
						01.24.12	2.47	0.65	1.55	0.17	0.01	0.03	0.06
11	Acute lymphoblastic leukemia	06.28.12	Lung biopsy	No fungal evidence	Probable	06.14.12	0.25	0.21	16.4	0.44	0.03	-‡	-‡
						07.10.12	61.97	1.24	2.28	1.36	0.12	0.08	0.05
						08.09.12	0.05	ANP	7.18	1.29	0.04	0.02	0.03
12	Acute lymphoblastic leukemia	ND	NA	NA	Probable	09.09.11	1.66	0.70	36.71	0.03	0	0.04	0.01
						09.20.11	2.18	0.85	12.96	0.10	0.04	0.04	0.03
13	Chronic lymphocytic leukemia	ND	NA	NA	Probable	08.23.10	4.83	0.04	0.07	No induction of CD154 expression due to nearly undetectable numbers of T cells			
14	Aplastic anemia	08.04.11	Lung biopsy	No fungal evidence	Probable	07.12.11	0.53	0.52	48.51	CD154 background expression too high to determine fungi-reactive T cell frequencies			
						07.22.11	0.79	0.74	45.58	CD154 background expression too high to determine fungi-reactive T cell frequencies			
15	Multiple myeloma	ND	NA	NA	Probable	07.07.11	1.16	0.34	1.21	CD154 background expression too high to determine fungi-reactive T cell frequencies			
						07.12.11	0.06	ANP	1.32	CD154 background expression too high to determine fungi-reactive T cell frequencies			
16	Acute myeloid leukemia	ND	NA	NA	Probable	05.12.11	0.49	0.44	32.18	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells			
17	Acute myeloid leukemia	ND	NA	NA	Possible	06.30.11	0.59	0.35	63.57	0.08	0	0	-†
						07.07.11	5.95	0.99	24.35	0.08	0	0.01	-†

						07.12.11	9.58	0.79	9.98	0.21	0.02	0.02	-†
18	Acute lymphoblastic leukemia	ND	NA	NA	Possible	08.18.11	3.40	0.34	17.41	0.16	0.02	0.02	0.02
19	Acute myeloid leukemia	ND	NA	NA	Possible	11.17.11	1.11	0.19	2.23	0.09	0.05	0	0.07
20	Acute lymphoblastic leukemia	ND	NA	NA	Possible	11.09.11	8.61	0.26	12.38	0.16	0.06	0	0
						11.18.11	8.10	0.79	4.12	0.06	0.04	0.04	0.02
21	Acute myeloid leukemia	ND	NA	NA	Possible	09.09.11	0.08	ANP	18.87	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells			
						09.20.11	0.17	0.15	26.6	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells			
22	T cell leukemia	ND	NA	NA	no	01.12.11	0.49	0.47	81.21	0.05	-†	-†	-†
23	Acute myeloid leukemia	ND	NA	NA	no	01.13.11	5.13	0.31	4.98	0.06	-†	-†	-†
						06.01.11	<0.05	ANP	5.12	No induction of CD154 expression due to nearly undetectable numbers of antigen presenting cells			
						06.16.11	2.35	0.66	2.09	0.19	0.02	0	-†
24	Acute myeloid leukemia	ND	NA	NA	no	06.01.11	0.08	ANP	11.35	0.05	-‡	-‡	-‡
25	Acute myeloid leukemia	ND	NA	NA	no	07.19.11	2.40	1.42	11.14	0.06	0	0	-†
						08.18.11	0.51	0.29	29.04	0.07	0.02	0.02	0.04
26	Allogeneic HSCT	ND	NA	NA	no	08.18.11	0.84	0.67	21.03	0.05	0.03	0.04	0.01
27	Allogeneic HSCT	ND	NA	NA	no	08.18.11	9.33	1.33	20.44	0.08	0.01	0.01	0.08
28	Non-Hodgkin's lymphoma	ND	NA	NA	no	09.20.11	9.33	2.11	3.13	0.04	0.01	0.02	0.02
29	Acute T cell leukemia	ND	NA	NA	no	01.12.11	6.19	0.46	3.75	0.09	-†	-†	-†
30	Acute lymphoblastic leukemia	ND	NA	NA	no	01.12.11	2.11	0.48	13.38	0.09	-†	-†	-†
						03.31.11	6.52	0.43	5.09	0.17	-†	-†	-†
31	Acute myeloid leukemia	ND	NA	NA	no	01.18.11	4.00	0.54	1.82	0.10	-†	-†	-†
32	Acute myeloid leukemia	ND	NA	NA	no	01.27.11	2.66	0.91	5.51	0.06	-†	-†	-†
33	T cell leukemia	ND	NA	NA	no	02.01.11	4.36	0.43	14.28	0.09	-†	-†	-†

						03.22.11	2.80	0.65	11.00	0.23	-†	-†	-†
34	Acute lymphoblastic leukemia	ND	NA	NA	no	02.08.11	1.16	0.22	17.13	0.04	-†	-†	-†
35	Acute myeloid leukemia	ND	NA	NA	no	02.08.11	3.11	0.84	17.01	0.08	-†	-†	-†
36	Acute lymphoblastic leukemia	ND	NA	NA	no	03.31.11	3.03	0.56	13.65	0.08	-†	-†	-†
37	Acute lymphoblastic leukemia	ND	NA	NA	no	04.05.11	4.51	0.77	7.38	0.09	-†	-†	-†
38	Hodgkin's lymphoma	ND	NA	NA	no	07.19.11	4.27	0.10	0.16	0.06	0.02	0.06	-†
39	Allogeneic HSCT	ND	NA	NA	no	07.26.11	8.44	2.20	25.33	0.22	0.04	0.05	0.02
40	CUP syndrome	ND	NA	NA	no	09.06.11	5.09	0.70	8.43	0.07	0.01	0.03	0
41	Acute myeloid leukemia	ND	NA	NA	no	09.09.11	4.94	0.54	1.93	0.10	0.13	0	0.03
						09.20.11	3.61	0.21	5.89	0.09	0.14	0.05	0.02
42	Acute myeloid leukemia	ND	NA	NA	no	01.25.11	8.40	0.84	10.52	0.65	-†	-†	-†
						02.25.11	7.67	1.56	9.14	0.28	-†	-†	-†
						03.22.11	9.52	1.20	9.11	0.31	-†	-†	-†
43	Acute myeloid leukemia	ND	NA	NA	no	08.26.11	2.93	1.37	11.10	0.21	0.38	0.28	0.06
						08.30.11	2.18	0.88	12.91	0.22	0.36	0.16	0.03
44	Non-Hodgkin's lymphoma	ND	NA	NA	no	09.06.11	0.06	ANP	0.09	No induction of CD154 expression due to nearly undetectable numbers of T cells			
45	Hodgkin's lymphoma	ND	NA	NA	no	08.30.11	1.22	0.02	0.15	No induction of CD154 expression due to nearly undetectable numbers of T cells			

* Time frame -1 day, if not performed on the day of blood sampling;

† Specific fungal lysate was not yet available at the time of blood sampling;

‡ Cell count too low to test against all fungal lysates;

WBC, white blood cell; PBMC, peripheral blood mononuclear cells; ND, not done; NA, not applicable, ANP, analysis not possible; HSCT, hematopoietic stem cell transplantation; CUP, cancer of unknown primary.

Table S2. Contingency table of all analyzed patient cases with evaluable T cell responses*

Diagnosis of pulmonary invasive mold infection according to EORTC/MSG consensus	Fungus-specific T cell frequencies		Total patient number
	Increased	Not increased	
Proven	6	0	6
Probable	5	1	6
Possible	0	4	4
No fungal infection	2	20	22
Total	13	25	**38**

P=0.0000007, odds ratio (95 %) =132 (10.79 – 1615.08); sensitivity =0.92 (0.597 – 0.996), specificity =0.92 (0.734 – 0.987); *, patients with low antigen presenting cell or T cell counts and undeterminable fungus-reactive T cell frequencies were excluded.

Table S3. Contingency table of all analyzed patients

Diagnosis of pulmonary invasive mold infection according to EORTC/MSG consensus	**Fungus-specific T cell frequencies**		**Total patient number**
	Increased	**Not increased**	
Proven	6	0	6
Probable	5	5	10
Possible	0	5	5
No fungal infection	2	22	24
Total	13	32	**45**

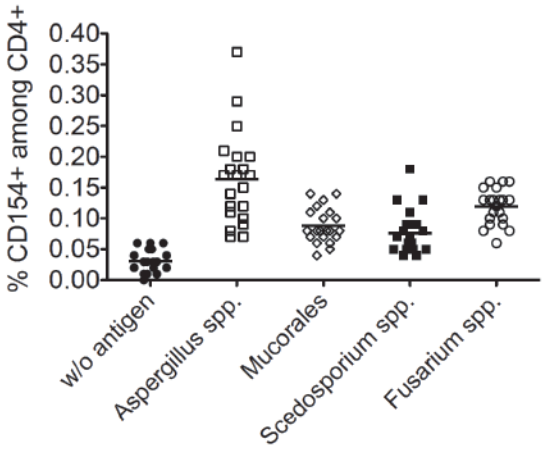
P=0.00003, odds ratio (95 %) =29.7 (4.99 – 176.72); sensitivity =0.69 (0.41 – 0.88), specificity =0.93 (0.76 – 0.99).

Table S4. Contingency table of patients with evaluable T cell responses*, except possible cases

Diagnosis of pulmonary invasive mold infection according to EORTC/ MSG consensus	Fungus-specific T cell frequencies		Total patient number
	Increased	Not increased	
Proven	6	0	6
Probable	5	1	6
No fungal infection	2	20	22
Total	13	21	**34**

P=0.000003, odds ratio (95 %) =110 (8.93 – 1354.51); sensitivity =0.92 (0.598 – 0.996), specificity =0.91 (0.69 – 0.98); *, patients with low antigen presenting cell or T cell counts and undeterminable fungus-reactive T cell frequencies were excluded.

Figure S1



Aspergillus spp. = *Aspergillus fumigatus, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus*

Mucorales = *Rhizopus oryzae, Lichtheimia corymbifera*

Scedosporium spp. = *Scedosporium apiospermum, Scedosporium prolificans*

Fusarium spp. = *Fusarium oxysporum, Fusarium solani, Fusarium verticillioides*

Figure S1. Frequencies of T cells from healthy donors after stimulation with pooled fungal lysates. Statistical analyses with horizontal lines indicate mean values (n=21).

Figure S2

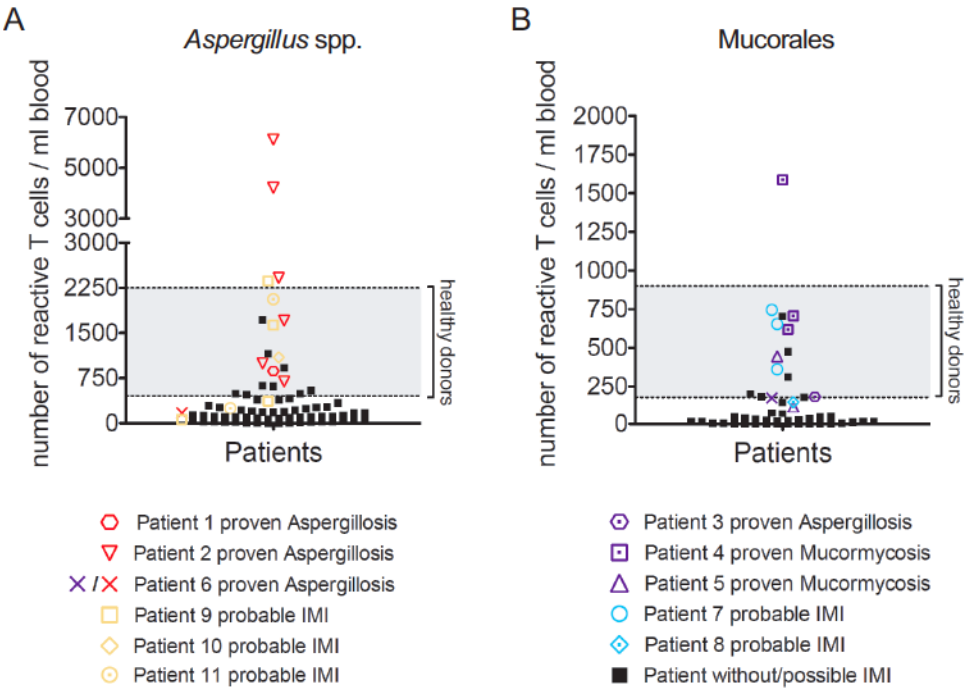


Figure S2. Absolute numbers of fungus-reactive CD4$^+$ T cells in peripheral blood. The absolute numbers of *Aspergillus* spp.- and Mucorales-reactive T cells were calculated based on the frequencies of mold-reactive CD154$^+$ T cells within CD4$^+$ lymphocytes and the CD4$^+$ lymphocyte count per mL of peripheral blood in high-risk patients without an invasive mold infection (black boxes; n=24) and with a proven or probable invasive mold infection according to EORTC/MSG consensus criteria. Patients with increased reactive T cell measurements for *Aspergillus* spp. are depicted as red/orange symbols (n=6, in total 38 patients analyzed) and/or for Mucorales as dark/light blue symbols (n=6, in total 23 patients analyzed). The range of healthy donors is marked in grey.

3.6 Manuscript VI

"Identification of Immunogenic Antigens from *Aspergillus fumigatus* by Direct Multi-Parameter Characterization of Specific Conventional and Regulatory $CD4^+$ T cells"

Bacher P, Kniemeyer O, Teutschbein J, Thoen M, Vödisch M, Wartenberg D, Scharf DH, Koester-Eiserfunke N, Schütte M, Dübel S, Assenmacher M, Brakhage AA, Scheffold A.

Manuscript submitted.

Identification of immunogenic antigens from *Aspergillus fumigatus* by direct multi-parameter characterization of specific conventional and regulatory CD4$^+$ T cells

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Short title: Immunogenic T cell antigens from *Aspergillus fumigatus*

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Abstract

$CD4^+$ T cells orchestrate immune responses against fungi such as *Aspergillus fumigatus,* a major fungal pathogen in humans. The complexity of the fungal genome and lifestyle questions the existence of one or a few immune dominant antigens and also complicates rational screening for immunogenic antigens useful for immunotherapy or diagnostics. Here we used a recently developed flow-cytometric assay for the direct *ex vivo* characterization of *A. fumigatus*-specific $CD4^+$ T cells for rapid identification of physiological T cell targets in healthy donors. We show that the T cell response is mainly directed against metabolically active *A. fumigatus* morphotypes and stronger against membrane protein fractions as compared to cell wall or cytosolic proteins. The further analysis of 15 selected single *A. fumigatus* proteins revealed a highly diverse donor- as well as protein-dependent reactivity pattern. Importantly, the parallel assessment of T cell frequency, phenotype and function allowed to differentiate between proteins eliciting strong memory T cell responses *in vivo versus* antigens that rather induce T cell exhaustion or no reactivity *in vivo*. The regulatory T cell (Treg) response mirrors the conventional T cell (Tcon) response in terms of numbers and target specificity. Thus our data reveal that the fungal T cell immunome is complex but the *ex vivo* characterization of reactive T cells allows to classify antigens and to predict potential immunogenic targets. Tcon responses are counterbalanced by a strong Treg response, suggesting that Treg depletion strategies may be helpful to improve anti-fungal immunity.

Introduction

Aspergillus fumigatus is a ubiquitous spore-producing mold that can cause a diverse spectrum of human diseases, ranging from allergic hypersensitivity and non-invasive colonization to life-threatening invasive infections. Invasive aspergillosis (IA) is the most devastating disease caused by this fungus in immunocompromised patients. Despite new anti-fungal drugs, morbidity and mortality continue to be unacceptable high and IA has become a major cause of infection-related mortality in hematopoietic stem cell recipients (1, 2).

Although we routinely inhale several hundreds or thousands of *A. fumigatus* conidia per day (3, 4), immune-competent individuals are efficiently protected by innate and adaptive immune mechanisms (5, 6). Lung-resident alveolar macrophages and neutrophils ingest and kill *A. fumigatus* conidia and germlings and recruit other immune cells by secretion of pro-inflammatory cytokines (7). There is increasing evidence that $CD4^+$ T cells orchestrate the anti-fungal immune response. In mouse models, monocytes and dendritic cells have been shown to prime *A. fumigatus*-specific $CD4^+$ T cell responses that migrate to the airways (8-10). Adoptive transfer of *A. fumigatus*-specific IFN-γ producing T cells protected mice from invasive fungal disease (11) and correlated with survival of IA patients (12). In accordance with the idea that humans are constantly confronted with fungal antigens we recently showed that a small population of *A. fumigatus*-specific T cells is indeed consistently present in healthy donors (13). In IA patients, the frequencies of *A. fumigatus*-reactive T cells are strongly increased (unpublished observation) indicating the involvement of specific $CD4^+$ T cells in antifungal immune defense.

Therefore, approaches supporting fungus-specific $CD4^+$ T cells in immuno-compromised persons, *e.g.* by vaccination or adoptive T cell transfer (14-17) seem to be promising for preemptive or therapeutic intervention against invasive fungal infections. However, in order to develop efficient immunotherapies, a crucial first step is to define the antigen specificity of

human $CD4^+$ T cells *in vivo*. Due to the complexity of the *A. fumigatus* proteome it is currently not known against which fungal antigens human T cells predominantly react and which T cell specificities are protective. In addition it is not clear, whether regulatory T cells (Treg), which represent the major part of the human T cell response against *A. fumigatus* (18) recognize the same or different antigens, as their conventional T cell (Tcon) counterparts. The *A. fumigatus* genome contains several thousand open reading frames, encoding potential antigenic proteins. The initially inhaled resting conidia convert into swollen conidia within 4-5 hours upon arrival in the lungs and further germinate to form germ tubes and later hyphae (19). Proteomic approaches have revealed differences of the most abundant proteins present in conidia and hyphae (20-22). Therefore, further variability of potential T cell antigens may result from the morphogenic status of the fungus at the time point of confrontation with the immune system, which might significantly impact the generation of a specific T cell response. Thus, the identification of the main *in vivo* T cell targets, *i.e.* proteins derived from the different morphogenic stages and subcellular structures, as well as knowledge about their Tcon or Treg activating potential, would have important implications on vaccination or adoptive cell therapy strategies.

We have recently described a highly specific and sensitive assay to enumerate and characterize antigen-specific $CD4^+$ T cells directly *ex vivo* based on $CD154^+$ pre-enrichment (Antigen-Reactive T cell Enrichment, ARTE) (13, 23). Here we used ARTE to systematically quantify and characterize the very rare human peripheral $CD4^+$ T cells reacting against crude lysates of *A. fumigatus,* as well as selected proteins or protein fractions. We found that the T cell response against *A. fumigatus* is highly diverse, questioning the existence of one or a few immunodominant *A. fumigatus* antigens. However, we identified quantitative and qualitative differences within the single protein-specific T cell populations, which may provide a general screening procedure to classify fungal antigens and identify potentially immunogenic *in vivo* targets.

Materials and Methods

Blood donors

Buffy coats from healthy donors were obtained from the Institute for Transfusion Medicine, University Hospital Dortmund after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare Life Sciences, Freiburg, Germany) density gradient centrifugation.

Preparation of A. fumigatus lysates

For the generation of all *A. fumigatus* (strain ATCC46645) protein extracts, except for the lysate of resting conidia (RC), $2x10^8$ conidia were inoculated in 200 ml YPD medium and cultured at 37 °C and 200 rpm. Swollen conidia (SC) were harvested after 6 h, germinated conidia (GC) after 8 h, and mycelium after 20 h of cultivation. Cells were recovered by centrifugation (RC, SC, GC) or filtration (mycelium) and washed with water before storage at -80 °C. Total RC, SC or GC lysates were generated by disruption of frozen cells in saline [0.9 % (w/v) NaCl] using a Micro-Dismembrator (Sartorius). For total mycelial lysate, frozen mycelium was ground in liquid nitrogen by using a mortar and pestle and resuspendend in PBS supplemented with 2 mM $MgCl_2$. Total soluble protein fractions of the lysates (crude lysates) were obtained after removal of insoluble material (cell wall pellet) by centrifugation for 15 min at 10,000 × g. Fractionated mycelial protein extracts were obtained by sequential centrifugation of total mycelial lysate. The cell wall protein fraction was processed by resuspension of the cell wall pellet (15 min of centrifugation at 10,000 ×g) in PBS/2 mM $MgCl_2$. By centrifugation of the crude mycelial lysate at 100,000 × g for 60 min, the cell membrane-enriched protein fraction (pellet) was separated from the cytosolic protein fraction. Cell membrane extract was generated by resuspension of the membrane pellet in PBS/2 mM $MgCl_2$.

Extracts enriched in GPI-anchored proteins and membrane fractions depleted in GPI-anchored proteins were generated as described in (24). Briefly, total mycelial lysate was generated by resuspension in 200 mM Tris-HCl pH 8, 50 mM EDTA, 1 mM PMSF. The membrane pellet was obtained by centrifugation steps as described above and resuspended in 50 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.5 mM PMSF, 1 mM DTT. After freezing at –80 °C, the membrane extract was thawed on ice, supplemented with 1% (w/v) ß-n-octylglucopyranoside and incubated for 1 h at room temperature. Following centrifugation (100,000 × *g*, 60 min, 4 °C), the supernatant was taken (protein-fraction enriched in GPI-anchored proteins). The pellet was resuspended in 25 mM Na-phosphate buffer (pH 7) and used as GPI-depleted membrane protein extract.

Generation of recombinant A. fumigatus proteins

The recombinant Crf2 (AFUA_1G16190) protein was generated as described in (25). The recombinant GliT protein (AFUA_6G09740) was produced in *E. coli* and purified as described in (26). The open reading frames of the genes s*cw4* (AFUA_6G12380), *aspf3* (AFUA_6G02280), *shm2* (AFUA_3G09320), *cpcB* (AFUA_4G13170) and *aspf22* (AFUA_6G06770) were amplified from ATCC46645 cDNA and cloned into the expression vector pET43.1H6 for recombinant expression as HIS-tagged proteins. For *pst1* (AFUA_6G10290), a truncated version was generated by omitting the sequence, which encodes the first 17 amino acids of the N-terminal secretion signal peptide. The truncated *pst1* cDNA and the ORF of triosephosphate isomerase TpiA (AFUA_5G13450) were cloned into a pMalC2HTEV vector (27) for recombinant production as N-terminal MBP-HIS-tagged proteins. After transformation of *E. coli* BL21(DE3), all proteins were expressed by auto-induction in Overnight Express Instant TB medium (Novagen) at 30 °C for 24 hours. *E. coli* cells were harvested by centrifugation, homogenized in TBS (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT, 1 mM PMSF) [Pst1, TpiA, AspF3], TBS complemented with 8 M urea

[Scw4], TBS complemented with 10 % (v/v) glycerol [AspF22], or phosphate buffer (50 mM Na-P pH 8, 300 mM NaCl, 1 mM DTT, 1 mM PMSF) [Shm2, CpcB] and lysed using an emulsiFlex-C5 high pressure homogenizer (Avestin). The coding sequence of the FG-GAP repeat protein (AGUA_1G04130), which was devoid of the sequence encoding the 25 amino acids N-terminal secretion signal peptide (Δ25FG-GAP) and the ORF of AspF2 (AFUA_4G09580), were amplified and cloned into the expression vector pPICZαB. After transformation into *Pichia pastoris* (X33), culturing of cells and induction of expression was conducted according to the Pichia Expression Kit manual (life technologies, Darmstadt, Germany). Culture supernatants, containing the secreted HIS-tagged recombinant proteins, were collected after 48 h of expression and diluted (1/5) into binding buffer (10 mM Na-P pH 7.4, 500 mM NaCl, 10 mM imidazole).

All proteins were purified by affinity chromatography using an Äkta explorer purification system (GE Healthcare). If necessary, an ion exchange column (SOURCE 15Q, GE Healthcare) was used for further purification. Generally, all buffer exchanges were conducted using HiPrep 26/10 desalting columns (GE Healthcare). All HIS-tagged proteins were applied to a Ni Sepharose 6 Fast Flow (GE Healthcare) column, and eluted with 250 mM imidazole. MPP-tagged proteins were loaded onto an Amylose Resin HF (New England Biolabs) column and eluted with 10 mM maltose. The MBP-HIS-tag of MBP-tagged proteins and the HIS-tag of AspF3 were cleaved using TEV-protease and removed by its binding to Ni Sepharose. After buffer exchange to 20 mM Tris-HCl pH 8 (CpcB, Pst1, TpiA) or 20 mM Tris-HCl pH 8.5, 6 M urea (Scw4), corresponding proteins were further purified by ion exchange chromatography using an NaCl gradient for elution. Scw4 was further purified by reversed phase chromatopraphy (Source 15RPC, GE Healthcare) after exchanging the buffer to 0.05% (v/v) trifluoroacetic acid, 10 % (v/v) acetonitril. Purified Scw4 was lyophilized and resolved in PBS. For all other purified proteins, the buffer was exchanged to PBS (FG-GAP, Pst1, AspF22, AspF2, GliT) or 0.9 % (w/v) NaCl (Shm2, CpcB, AspF3, TpiA).

Stimulation of antigen-reactive T cells

PBMCs were resuspended at a concentration of 1×10^7/ml in RPMI-1640 (Miltenyi Biotec, Bergisch Gladbach, Germany), supplemented with 5 % (v/v) human AB-serum (BioWhittaker/Lonza, Walkersville, MD, USA), and 2 mM L-glutamine (PAA Laboratories, Pasching, Austria). Cells were stimulated for 7 hours with the following antigens: *A. fumigatus* lysates (each 40 µg/ml), *C. albicans*-lysate (20 µg/ml; Greer Laboratories, Lenoir, NC, USA), CMV-lysate (10 µg/ml, Siemens Healthcare Diagnostics, Marburg, Germany), recombinant *A. fumigatus* proteins (Crf2, Pst1, Aspf2, Aspf3, Shm2, FG-GAP, GliT, Aspf22, CpcB, TpiA, Scw4; each 20 µg/ml) or peptide pools (*C. albicans* MP65, Gel1, Crf1, Aspf3, CatB, Sod3, Shm2; each 0.6 nmol/peptide/ml; all from Miltenyi Biotec), or pools of proteins according to the classification into immunogenic (Scw4, CRF1, CRF2, Pst1, Shm2, each 20 µg/ml), non-target (Gel1, CatB; each 20 µg/ml) and exhausted (Aspf2, CpcB, Aspf3, FG-GAP; each 20 µg/ml). 1µg/ml CD40 and 1µg/ml CD28 functional grade pure antibody (both Miltenyi Biotec) was added. In some experiments, $CD45RA^+$ cells were depleted from PBMCs prior stimulation using CD45RA microbeads and LD columns (Miltenyi Biotec).

Enrichment and characterization of antigen-reactive T cells

Enrichment of reactive $CD154^+$ T cells or combined enrichment of CD154+/CD137+ T cells was performed using the CD154 MicroBead Kit alone, or in combination with the CD137 MicroBead Kit (both Miltenyi Biotec). In brief, cells were indirectly magnetically labeled with CD154-Biotin and CD137-PE followed by anti-Biotin Microbeads and anti-PE Microbeads and enriched by two sequential MS MACS columns (Miltenyi Biotec). For analysis of cytokine expression, 1 µg/ml Brefeldin A (Sigma Aldrich) was added for the last 2 hours of stimulation. Surface staining was performed on the first column, followed by fixation, permeabilization (Inside stain Kit; Miltenyi Biotec) and intracellular cytokine

staining on the second column, as described in (13), or staining of Foxp3 using the Foxp3 Staining Buffer Set (Miltenyi Biotec).

In vitro expansion and re-stimulation of antigen-reactive T cell lines

Magnetically enriched $CD154^+$ T cells were expanded with 1:100 mitomycin C (Sigma Aldrich) treated autologous feeder cells in X-Vivo™15 (BioWhittaker/Lonza), supplemented with 5 % (v/v) AB-serum (BioWhittaker/Lonza), 200 U/ml IL-2 (Proleukin®; Novartis, Nürnberg, Germany) and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Antibiotic Antimycotic Solution, Sigma Aldrich) at a density of $2.5x10^6$ cells/cm^2. During expansion for 2-3 weeks, medium was replenished and cells were split as needed.

5×10^5 expanded T cells were re-stimulated with autologous CD3-depleted (CD3 MicroBeads; Miltenyi Biotec) PBMC in a ratio of 1:1 in 96-well flat bottom plates with different antigens in presence of 1µg/ml CD28 functional grade pure Ab for 2h plus additional 4h with 1µg/ml Brefeldin A (Sigma Aldrich). After fixation and permeabilization cells were stained intracellularly for CD154 and cytokines.

Flow cytometry

Different combinations of the following monoclonal antibodies were used according to manufacturer´s recommendations: CD4-VioBlue, CD4-FITC, CD4-APC-Vio770 (VIT4), CD3-APC (BW264/56), CD14-VioGreen, CD14-PerCP (TÜK4), CD20-VioGreen, CD20-PerCP (LT20), CD8-VioGreen (BW135/80), CD45RO-FITC, CD45RO-PerCP (UCHL-1), CCR7-PE (REA108), CD45RA-APC, CD45RA-FITC (T6D11), anti-Biotin-PE, anti-Biotin-VioBlue (Bio3-18E7), CD154-PE, CD154-APC, CD154-VioBlue (5C8), TNF-α-FITC, TNF-α-PE-Vio770 (cA2), IFN-γ-FITC, IFN-γ-APC, IFN-γ-PE (45-15), IL-2-APC (N7.48A), IL-17-FITC, IL-17-PE (CZ8-23G1), IL-10-PE (B-T10), IL-4-PE (7A3-3), CD137-PE (4B4-1)

(all Miltenyi Biotec), CD45RO-PE.Cy7 (UCHL-1, BD Bioscience, San Jose, CA, USA), IFNγ-PerCP-Cy5.5 (4S.B3; BioLegend, San Diego, CA, USA), IL-22-PE (142928; R&D Systems Europe, Ltd., Abingdon, UK), Foxp3-PerCP-Cy5.5 (PCH101; eBioscience, San Diego, CA, USA). Data were acquired on a MACSQuant® analyzer and MACSQuantify™ software was used for analysis (both Miltenyi Biotec).

Statistical analysis

Statistical tests were performed with GraphPad PRISM® software 5.0 (GraphPad Software, La Jolla, CA, USA) using two-tailed paired Student´s *t*-test. *P* values of < 0.05 were considered statistically significant.

Results

Human CD4$^+$ T cell response against lysates of different *A. fumigatus* morphotypes

CD4$^+$ T cells specifically reacting against *A. fumigatus* can be identified using CD154 expression as a specific read-out for antigen activated CD4$^+$ T cells after short *in vitro* stimulation with fungal lysate (13, 23). To analyze against which *A. fumigatus* morphotype the human T cell response is directed, peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated for 7 hours with crude lysates from resting, swollen and germinating conidia or mycelia, containing the total soluble fraction of the mechanically disrupted fungal cells. Reactive CD154-expressing CD4$^+$ T cells were identified by flow-cytometry. Although all lysates induced a small population of CD154 expressing CD4$^+$ T cells, the frequency of reactive cells stimulated with resting conidia lysate was significantly lower, compared to stimulation with lysates from other morphotypes (Fig. 1A, B). To enable the direct *ex vivo* phenotypic and functional characterization of the specific T cells, we next magnetically pre-enriched the rare antigen-reactive CD154$^+$ T cells from larger cell numbers (1×10^7 PBMC) (13). Again, a significantly higher number of target cells could be detected after stimulation with lysates of the more progressed development stages *versus* the resting conidia lysate (Fig. 1C). However, analysis of cytokine production and phenotype revealed no major differences of T cells stimulated with the different *A. fumigatus* morphotype lysates: as shown in Fig. 1D, against all lysates a high frequency of TNF-α and IL-2 producers could be detected, whereas the production of the lineage defining cytokines IFN-γ and IL-17 was only low, although IFN-γ production was clearly predominant over IL-17, as already described before (13, 28, 29). Similarly, irrespective of the *A. fumigatus* lysate used for stimulation a comparable amount of reactive memory T cells was detected (Fig. 1E).

In summary, these results demonstrate that reactive memory CD4$^+$ T cells against different morphotypes of *A. fumigatus* are present in healthy human donors and suggest that the strongest T cell response is directed against the actively growing fungus.

Swollen and germinating conidia as well as mycelia contain overlapping T cell antigens

We next addressed the question, whether the reactive $CD4^+$ T cells recognize different or the same antigens expressed by the various *A. fumigatus* morphotypes. To this end, specific T cell lines were generated by expanding the magnetically enriched $CD154^+$ T cells after stimulation with the different morphotype lysates. Upon re-stimulation the T cell lines initially stimulated with protein extracts of swollen conidia, germinating conidia and mycelia were equally reactive to either lysate, as shown by re-expression of CD154 and production of cytokines (Fig. 2A, B). However, the re-stimulation with resting conidia lysate was in each case significantly lower, suggesting that a considerable proportion of T cell antigens, which are present in the metabolically active morphotypes (swollen and germinating conidia, mycelia), are missing in the resting conidia lysate. Furthermore, resting conidia-reactive T cell lines reacted equally well to re-stimulation with each lysate, suggesting that resting conidia do not contain a significant fraction of T cell target proteins solely present in the dormant stage. As expected, none of the expanded cell lines reacted upon re-stimulation with CMV-lysate as a control antigen, providing evidence for the specificity of the expanded fungus-reactive T cell lines. As for the *ex vivo* response, we observed no differences in the cytokine producing capacities of the different T cell lines upon re-stimulation (Fig. 2C).

A large proportion of the human *A. fumigatus*-specific T response is elicited by mycelial membrane proteins

Since the re-stimulation of the expanded *A. fumigatus*-reactive T cell lines revealed a comparable and largely overlapping T cell antigen repertoire of swollen and germinating conidia or mycelial lysate, we focused on the mycelial morphotype for further experiments. As the very first, the immune system encounters *A. fumigatus* protein antigens located on the cell surface, which include secreted, cell wall and membrane proteins. We therefore analyzed, whether the antigen-specific T cell response differs in response to distinct subcellular fractions of *A. fumigatus* mycelia. Lysates were generated from the mycelial cell wall,

membrane or cytosolic fraction and compared to the crude mycelial extract, as applied in the above described experiments. Interestingly, *ex vivo* stimulation of PBMCs with the cell wall or cytosolic fraction was significantly lower, compared to the crude mycelia lysate. In contrast, stimulation with the membrane fraction yielded similar frequencies and numbers of $CD154^+$ cells compared to the crude extract (Fig. 3A, B). However, we again observed no major differences in the *ex vivo* memory phenotype of T cells stimulated with the different cellular fractions (Fig. 3C), indicating the existence of a specific human memory T cells against components from all the different mycelial compartments.

To further corroborate the finding that a large proportion of the specific T cell repertoire against *A. fumigatus* is directed against mycelial membrane proteins, cell lines were expanded from initially crude extract or membrane fraction stimulated $CD154^+$ T cells. Re-stimulation confirmed, that the highest reactivity was obtained with lysate of the membrane fraction, whereas re-stimulation with the cell wall or cytosolic fraction was significantly lower (Fig. 3D, E).

A number of glycosylphosphatidylinositol (GPI) anchored proteins have been identified in the *A. fumigatus* membrane (24, 30, 31), some of which play a role in the organization of the fungal cell wall. Interestingly, some of these proteins have been described as fungal antigens (32). To further dissect the T cell response against the *A. fumigatus* membrane, we investigated the stimulatory capacity of a protein fraction enriched in GPI-anchored proteins and the remaining membrane fraction after the release of GPI-anchored proteins in the supernatant by the activity of endogenous phospholipases as described in (24). Interestingly, both fractions induced comparable frequencies of $CD154^+$ memory T cells after *ex vivo* stimulation of PBMCs (Supplementary Fig. S1A, B). In addition, re-stimulation of total mycelia-reactive T cell lines revealed similar reactivity for each fraction (Supplementary Fig. S1C, D), indicating that both, a fraction enriched in GPI-anchored proteins, as well as the membrane fraction depleted in GPI-anchored proteins, contain a considerable proportion of stimulatory T cell antigens.

In summary, these results revealed a broad and variable T cell response against different morphotypes and cellular fractions of *A. fumigatus*, but identified the mycelial membrane proteins as a major target of the *A. fumigatus*-specific T cell response in healthy human donors.

ARTE allows direct characterization of human CD4$^+$ T cells reacting against single *A. fumigatus* proteins

Until now, only few single proteins of *A. fumigatus* have been analyzed and directly compared in their capacity to elicit CD4$^+$ T cell responses in humans (29, 33, 34). In particular the direct qualitative and quantitative characterization of the responding T cells is missing, which avoids an experimental bias due to prolonged *in vitro* culture. However, the quality of the T cell response generated *in vivo* may provide important insight into the immunogenic properties of specific antigenic proteins.

Therefore, we performed multi-parameter analysis of the T cells specific for a panel of 15 selected *A. fumigatus* proteins with different biological functions and cellular localization. Either recombinant proteins or synthesized 15mer peptide pools covering the complete protein sequence were used for stimulation. The analyzed proteins included cell wall, GPI-anchored, secreted, as well as cytosolic proteins and were chosen based on their high abundance within the conidial, mycelial or secreted proteome and/or their previous description as being immunogenic, based on T cell or serum reactivity (Table 1). A concentration of 20 µg/ml of recombinant proteins for the stimulation of 1×10^7 PBMCs was determined based on titration of the single proteins on expanded total mycelia-reactive T cell lines (Supplemental Fig. S2). The *C. albicans* protein MP65 had previously been described as a major antigen target of human T cell responses (35, 36) and served as a positive control.

Against the majority of the analyzed single *A. fumigatus* proteins no reactive CD4$^+$ T cells above background could be detected by standard flow-cytometry without pre-enrichment (data not shown). To enable the direct *ex vivo* detection of reactive CD4$^+$ T cells against the

single *A. fumigatus* proteins, we performed ARTE from 1×10^7 stimulated PBMCs. Although the frequencies upon single protein stimulation were significantly lower as upon stimulation with *A. fumigatus* or *C. albicans* crude lysates, specific T cells against single proteins could be clearly detected compared to the non-stimulated control (Fig. 4A). The specificity of the *ex vivo* detected single protein-reactive $CD154^+$ T cells was confirmed by expansion and re-stimulation of specific T cell lines (Supplemental Fig. S3).

Interestingly, the T cell responses against the different proteins were quite variable with frequencies ranging from 1.2×10^{-6} to 3.1×10^{-4} (Fig. 4B) and showed strong intra- and inter-donor variability (Fig. 4A, B). As expected, this indicates an overall diverse repertoire of *A. fumigatus*-reactive $CD4^+$ T cells, probably due to different exposure and/or HLA-restriction. The subcellular location of the proteins did not result in a clear-cut phenotypic/functional characteristic of the resulting T cell response, although a trend towards a strong reactivity against membrane-associated proteins was observed.

Integration of phenotypic and functional markers of specific T cells allows classification of antigenic proteins

Since our method allows multi-parameter characterization of very rare single *A. fumigatus* protein-specific T cells, we integrated cytokine production, as well as phenotypic T cell markers, into our further analyses (Fig. 5). The combination of frequencies, naive/memory distribution and effector cytokine production allowed classification of the fungal proteins into three groups: "Immunogenic" proteins are characterized by high overall T cell frequencies, mainly memory type cells and high IFN-γ and/or IL-17 production. In contrast, "exhausting" proteins were classified due to their low to intermediate overall frequencies, and lack of effector cytokine production, although the majority of cells had a clear memory phenotype. These properties are indicative for deletion and/or anergy of specific T cells. These two groups with obvious immune reactivity *in vivo* contrast with the third group, which we termed

"non-target" proteins, since they induce high overall T cell frequencies, but strikingly a large proportion of the cells is still in the naive state and also lacks effector cytokine production. This finding indicates that no immune reactivity is induced *in vivo*. Interestingly, the reactivity against the mycelial crude lysate as shown in figure 5 is also characterized by a high frequency of naive T cells and rather low effector cell frequencies, when compared to the immunogenic protein group, suggesting that a large fraction of the fungal proteins actually belongs to the exhausting or non-target group.

T cell responses to single *A. fumigatus* proteins account only for a small fraction of the total response against *A. fumigatus* crude lysate in healthy donors

To confirm our hypothesis regarding antigen classification and to estimate to which extent the identified immunogenic proteins contribute to the total response *in vivo*, we generated specific T cell lines from pre-sorted $CD4^+CD45RO^+$ memory T cells after stimulation with crude mycelia lysate. The cell lines were then re-tested for their specificity by antigen stimulation with the crude lysate or the single *A. fumigatus* proteins, as well as a pool thereof (Fig. 6). In line with our hypothesis, the main reactivity within the memory cell line was indeed directed against the immunogenic proteins, whereas exhausting or non-target proteins had only minimal contribution, except for the FG-GAP repeat protein, which also generated low reactivity in some donors. However, for most proteins, including the immunogenic fraction, less than 1-2% of the expanded $CD4^+$ T cell lines responded upon re-stimulation with the single proteins. In addition, a high inter-donor variability of antigenic targets was observed. However, for some donors, reactivity against certain single proteins exceeded the 1-2% margin. This was especially true for the known antigen Aspf22, which generated strong responses in the majority of donor cell lines (mean reactivity 3.6%; range 0-18%).

Taken together, we showed that the human $CD4^+$ T cell response against *A. fumigatus* is highly heterogeneous and directed against a large number of different proteins and epitopes, questioning the existence of one or few immune dominant proteins. However, the direct

multi-parameter analysis of reactive T cells against the single *A. fumigatus* proteins allows predicting the immunogenic potential of a particular protein *in vivo* via integration of frequency, phenotype and function of specific T cells.

A. fumigatus-specific Tcon and Treg recognize the same antigens

We recently demonstrated that *A. fumigatus* surprisingly generates a strong Treg response *in vivo,* which even exceeds conventional memory T cells (Tmem) (18). We therefore analyzed whether the same or different antigens are recognized by *A. fumigatus*-specific Tmem and Treg. To this end, the single proteins were pooled according to our previous classification into an immunogenic group, containing proteins with the highest reactivity (Scw4, Crf1, Crf2, Pst1 and Shm2) as well as a non-target (Gel1, CatB) and exhausting (Aspf2, Aspf3, CpcB, Fg-Gap) group and used for stimulation in comparison to the mycelial crude lysate or the mycelial membrane fraction. CD137 which is expressed by Treg after 6 hours of stimulation was used together with CD154 enrichment to differentiate between Treg ($CD137^+CD154^-$) and Tcon ($CD137^-CD154^+$) (18, 37). As shown in figure 7A and B the Treg response mirrored the response of the Tcon, in that a high reactivity of specific $CD137^+$ Treg was found in response to the *A. fumigatus* crude lysate, as well as the membrane fraction and the immunogenic protein pool. Again, the majority of reactive $CD154^+$ Tcon against the immunogenic and exhausting protein pools displayed a memory phenotype, whereas a larger proportion against the non-target pool was still in the naive state (Fig. 7C). This resulted in an equally high Treg to Tmem ratio for all fractions (Fig. 7D), indicating that *A. fumigatus*-specific Treg and Tmem are directed against the same target antigens and that the *A. fumigatus*-specific T cell response is balanced by specific Treg cells.

Discussion

Here, we show that ARTE can be used for the direct quantification and multi-parameter characterization of rare human $CD4^+$ T cells specific for various antigens of the important human-pathogenic fungus *A. fumigatus*. The sensitivity and flexibility of the method enabled the analysis of T cells specific for various developmental stages, subcellular compartments as well as a large set of selected single recombinant proteins. Importantly, the multi-parameter characterization of T cells reactive against single *A. fumigatus* proteins allowed the classification of proteins into immunogenic, exhausting or non-target subgroups. Furthermore, our results revealed that the $CD4^+$ T cell response is directed against a broad panel of proteins present in metabolically active fungal morphotypes and that specific Tcon and Treg cells are elicited *in vivo* against the very same antigens.

The presence of *A. fumigatus*-specific $CD4^+$ T cells in human blood has been described in several studies, using *in vitro* stimulation assays with whole conidia and hyphae, crude lysates, single proteins or epitopes (13, 14, 28, 29, 33, 34, 38, 39). However, it has not been defined yet, which developmental stage (resting, swollen, germinating conidia or mycelia) and which subcellular protein fraction prime *A. fumigatus*-specific T cells in healthy human donors. Here, we demonstrate that the activated developmental stages of the fungus (swollen, germinating conidia and hyphae) contain the largest reservoir of potential T cell epitopes. Furthermore, T cell antigens in the metabolic active *A. fumigatus* morphotypes were largely overlapping, which is in line with recent results on the proteomic signature of *A. fumigatus* during early development. These studies showed that the majority of mycelial proteins are also present in all early, metabolically active morphotypes and only the abundance varies (21, 40, 41).

The further subcellular dissection of the *A. fumigatus* mycelia antigens showed that the membrane-bound proteins elicited the strongest T cell reactivity as compared to the cytosolic fraction. This result was corroborated by the analysis of T-cell reactivity against several single *A. fumigatus* proteins. Here, we observed a uniformly high frequency of reactive memory-

type T cells against all analyzed GPI-anchored proteins, except of Gel1, whereas the "exhausting" and "non-target" groups mainly consist of cytosolic or secreted proteins.

Indeed, the possibility to analyze the rare T cells reacting against single proteins highlights the potential of the ARTE technology, since it allows classifying proteins according to their *in vivo* antigenic potential. So far *A. fumigatus* $CD4^+$ target proteins have mainly been identified indirectly via the presence of isotype-switched antibodies (25, 32, 42-46). Alternatively, functional T cell assays such as proliferation or cytokine production have been used in a few studies (29, 33, 34, 38, 39, 47, 48), which do not allow quantitative enumeration and phenotypic characterization of the reactive T cells. In fact, for the majority of proteins analyzed, the frequencies of reactive T cells were $<10^{-4}$, which would not be detectable by standard methods. By the parallel assessment of T cell frequency, phenotype and function, three groups of *A. fumigatus* proteins could be defined: (i) immunogenic proteins, elicited a strong memory response and pro-inflammatory cytokine production in the majority of donors analyzed. (ii) In stark contrast, other non *in vivo* target proteins elicited a strong *in vitro* T cell reaction, but the specific T cells were mainly in a naive state, suggesting that the antigen is not presented *in vivo*. (iii) Finally, a large fraction of the analyzed proteins belonged to a third group, which we termed exhausting, since the overall frequencies, as well as effector cytokine production was relatively low, although the majority of reactive cells had a clear memory phenotype. This indicates that confrontation with the antigen takes place *in vivo* but rather induces anergy or deletion of the reactive T cells. Thus, the enumeration and multi-parameter characterization of T cells specific for single *A. fumigatus* proteins identifies protein-specific reactivity patterns and thus represents an important complementation to the data obtained with mixed antigen preparations.

From the identified immunogenic proteins, Crf1, Sod3 and Aspf22 have previously been described to elicit $CD4^+$ T cell responses in humans (29, 33, 34, 38). However, our analysis also identified new immunogenic proteins, including Scw4, Crf2 (25), Pst1, Shm2, GliT and TpiA that have not been described as human T cell targets before. The gliotoxin oxidase GliT

has recently been identified via an immunoproteome screening approach and has been suggested to represent a novel antigen for serologic diagnosis of aspergillosis (45). Interestingly also two other "immunogenic" proteins, the enolase Aspf22 and Shm2 were detected in immunoblots using sera from patients with allergic bronchopulmonary aspergillosis (49). In addition, Shm2 belongs to the most abundant proteins identified in the mycelial proteome (22).

It is important to note that the same characteristics also apply to other proteins that were tested in our study, e.g. CpcB, Aspf2 and Aspf3 (Table 1), which all belong to the "exhausting" group. Furthermore, CatB classified by our analysis as a "non-target" protein has previously been described to induce strong T cell proliferation *in vitro* but vaccination with CatB did not protect mice from invasive aspergillosis (33). Thus it is obvious, that other factors than protein localization, abundance or antibody reactivity are critical parameters to determine the true *in vivo* T cell stimulatory capacity and highlights the potential of our approach to systematically predict immunogenic and potential protective target proteins.

In addition to the phenotypic characteristics, ARTE also allows to determine the functionally important production of effector cytokines, such as IFN-γ or IL-17. Although IL-17 is frequently claimed as an important cytokine for anti-fungal immune responses, the importance for protection against fungal infections *versus* immunopathology is currently under debate (50-54). In this study we observed a predominant IFN-γ production and only low IL-17, which is in line with previous reports (13, 28, 29, 34) suggesting that *A. fumigatus* elicits predominantly Th1 responses *in vivo*. Th2 cytokines (IL-4, IL-5, IL-13) were only marginal produced against the defined pools of single proteins (data not shown) and have previously been shown to be typically below 5% of all reactive $CD154^+$ cells against the total soluble lysate (13). However, when single proteins were analyzed strong IFN-γ and IL-17 cytokine production was only observed against proteins classified as "immunogenic". Also against the immunogenic proteins, IFN-γ was the dominating cytokine confirming also on the

level of single proteins that the *in vivo* response against *A. fumigatus* is rather biased towards a Th1 pattern. Moreover, some proteins (*e.g.* Scw4, Pst1, GliT, Aspf22) elicited in addition to IFN-γ the co-production of relatively high amounts of IL-17. The knowledge about the specific cytokine induction potential of certain proteins may help to improve vaccine design in the future. However, the functional importance of the various T cell cytokines has to be determined beforehand. Interestingly, by pooling proteins according to our classification, we found that not only conventional memory T cells, but also regulatory T cells were strongly activated by the immunogenic proteins, indicating that Tcon and Treg recognize the same antigens. In fact the number of Treg exactly paralleled the number of memory Tcon resulting in a stable Treg/Tmem ratio for all antigens. This finding indicates that Treg are not selectively generated against a subgroup of proteins but their expansion seems to be coupled to the expansion of conventional T cells, which may be mediated via growth factor supply, such as IL-2 (55). Thus, the T cell response against all *A. fumigatus* proteins is controlled by Treg and therefore the depletion of Treg might be a promising strategy for releasing full T cell responses, *e.g.* for immunotherapeutic approaches.

Finally, despite the fact that our analysis could define a set of immunogenic *A. fumigatus* proteins, the overall T cell response was directed against a multitude of different proteins. In addition, the T cell frequencies against single antigens were very low and there was significant donor-to-donor variation. This indicates that the *A. fumigatus*-specific T cell response is largely heterogeneous and also determined by host-specific or environmental factors, such as MHC restriction or variability in timing and dosage of antigen exposure. In line with this, a recent study identified 7 and 30 different T cell epitopes within the Crf1 and CatB protein, respectively, which are presented by different HLA-class II molecules (34). Thus, the existence of a single or even a few immune dominant antigens is rather unlikely emphasizing even more the importance of technologies allowing the fast *ex vivo* classification of single proteins or even peptides in individual donors, as demonstrated here.

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Conflict of Interest

MA works as an employee of Miltenyi Biotec and AS works as a consultant for Miltenyi Biotec. All other authors declare no financial conflicts of interest.

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Figure legends

Figure 1. Memory $CD4^+$ T cells from healthy human donors show specific reactivity against lysates from different *A. fumigatus* growth phases. Following stimulation of PBMCs with the indicated crude lysates of different morphotypes, $CD154^+$ expression on $CD4^+$ T cells was analyzed directly *ex vivo*. (A) Cells were gated on lymphocytes and aggregates (scatter area *versus* height), dead cells and non-T cell lineages ($CD14^+$, $CD20^+$, dump) were excluded. Representative dot plot examples from one donor with frequencies of $CD154^+$ cells among $CD4^+$ lymphocytes and (B) summary of several donors with horizontal lines indicating mean values. (C-E) $CD154^+$ cells were magnetically pre-enriched and stained for cytokine expression and phenotypic markers. (C) Number of $CD154^+$ cells obtained from 1×10^7 stimulated PBMCs. (D) Percentages of cytokine-expressing cells among $CD154^+$ T cells and (E) percentages of $CD45RO^+$ memory cells among $CD154^+$ T cells are shown. Significance was determined using paired Student´s *t*-test. RC = resting conidia; SC = swollen conidia; GC = germinating conidia.

Figure 2. Resting conidia contain less T cell antigens than other *A. fumigatus* morphotypes. Following stimulation with the different crude growth phase lysates, $CD154^+$ cells were magnetically isolated and expanded for 2 weeks. Upon re-stimulation with and without antigens as indicated, reactive $CD4^+$ T cells were determined by CD154 and TNF-α expression. (A) Representative dot plot examples from one donor with percentage of reactive cells gated on $CD4^+$ lymphocytes and (B) statistical analysis of several donors with horizontal lines indicating mean values. (C) The fungal lysate reactive T cell lines were re-stimulated with the specific lysate used for initial stimulation and analyzed for intracellular cytokine expression. Percentages of cytokine producing cells among $CD154^+$ cells are depicted. Significance was determined using paired Student´s *t*-test. RC = resting conidia; SC = swollen conidia; GC = germinating conidia.

Figure 3. A high proportion of stimulatory T cell antigens are located in the fungal membrane. (A-C) PBMCs were stimulated with the indicated lysates and analyzed *ex vivo* for $CD154^+$ expression among $CD4^+$ lymphocytes. (A) Frequencies of reactive $CD154^+$ cells among $CD4^+$ and (B) enriched $CD154^+$ cell numbers following magnetic isolation from 1×10^7 PBMCs. (C) Percentages of $CD45RO^+$ memory cells among $CD154^+$ T cells are shown. (D, E) Specific T cell lines were expanded from isolated $CD154^+$ T cells and analyzed for reactivity by antigen re-stimulation. (D) Representative dot plot examples from one donor and (E) summary for several donors. Horizontal lines indicate mean values. Significance was determined using paired Student´s *t*-test.

Figure 4. *Ex vivo* enumeration of $CD4^+$ T cells reactive against single *A. fumigatus* proteins. 1×10^7 PBMCs were stimulated with *A. fumigatus* crude mycelia lysate, *C. albicans* lysate and MP65 as control antigens, or single *A. fumigatus* proteins as indicated. $CD154^+$ cells were enriched and stained intracellularly for cytokine expression. (A) Representative dot plot examples from one donor. For an optimal detection of rare $CD154^+$ events, aggregates, dead cells and non-target cells ($CD8^+$, $CD14^+$, $CD20^+$) were excluded by using a dump channel. The numbers of $CD154^+$ cells obtained after enrichment are indicated. (B) Enumeration of reactive $CD4^+$ T cells in several donors. The total number of $CD154^+$ cells obtained after enrichment was normalized to the total number of $CD4^+$ cells applied to the column. Background enriched from the non-stimulated control was subtracted. pp = peptide pool; r = recombinant

Figure 5. Combined characterization of frequency, phenotype and function enables classification of antigenic proteins. Enriched $CD154^+$ cells were *ex vivo* analyzed for frequency, expression of CD45RO and pro-inflammatory cytokine production and classified into “immunogenic”, “non-target” and “exhausting” proteins, as indicated. Frequency was

determined as in Fig. 4, percentages of $CD45RO^+$ memory cells among $CD154^+$ cells and percentages of cytokine-expressing cells among $CD154^+$ T cells are shown. pp = peptide pool; r = recombinant

Figure 6. Contribution of single *A. fumigatus* protein-reactive $CD4^+$ T cells to the total *A. fumigatus* lysate response. 1×10^7 CD45RA-depleted PBMCs from healthy donors were stimulated with crude mycelia lysate. $CD154^+$ cells were enriched and expanded for 2 weeks. Expanded cell lines were re-stimulated with crude mycelia lysate or indicated proteins in presence of autologous APCs and the reactive $CD4^+$ T cells were determined by CD154 and TNF-α expression. Summary of reactive $CD4^+$ T cells from several donors with mean values is shown (n = 16). Background from non-stimulated sample was subtracted.

Figure 7. Stimulation of *A. fumigatus* specific Treg with pools of immunogenic, non-target and exhausting proteins. 2×10^7 PBMCs were stimulated with *A. fumigatus* crude mycelia lysate, membrane lysate or the indicated pools of single proteins. $CD154^+$ and $CD137^+$ cells were magnetically enriched and stained for Foxp3 expression. (A) Representative dot plot examples from one donor. Cells are gated on $CD4^+CD154^-$ lymphocytes and Foxp3 expression on $CD137^+$ cells is depicted. The numbers of $CD137^+Foxp3^+$ Treg cells obtained after enrichment are indicated. (B) Enumeration of reactive $CD154^+$ Tcon and $CD137^+$ Treg in several donors (n = 6). The total number of $CD154^+$ and $CD137^+$ cells obtained after enrichment was normalized to the total number of $CD4^+$ cells applied to the column. Background enriched from the non-stimulated control was subtracted. (C) Percentages of $CD45RO^+$ memory cells among $CD154^+$ cells (Tmem). (D) Ratio of $CD137^+$ Treg to $CD154^+CD45RO^+$ Tmem.

Figure 1

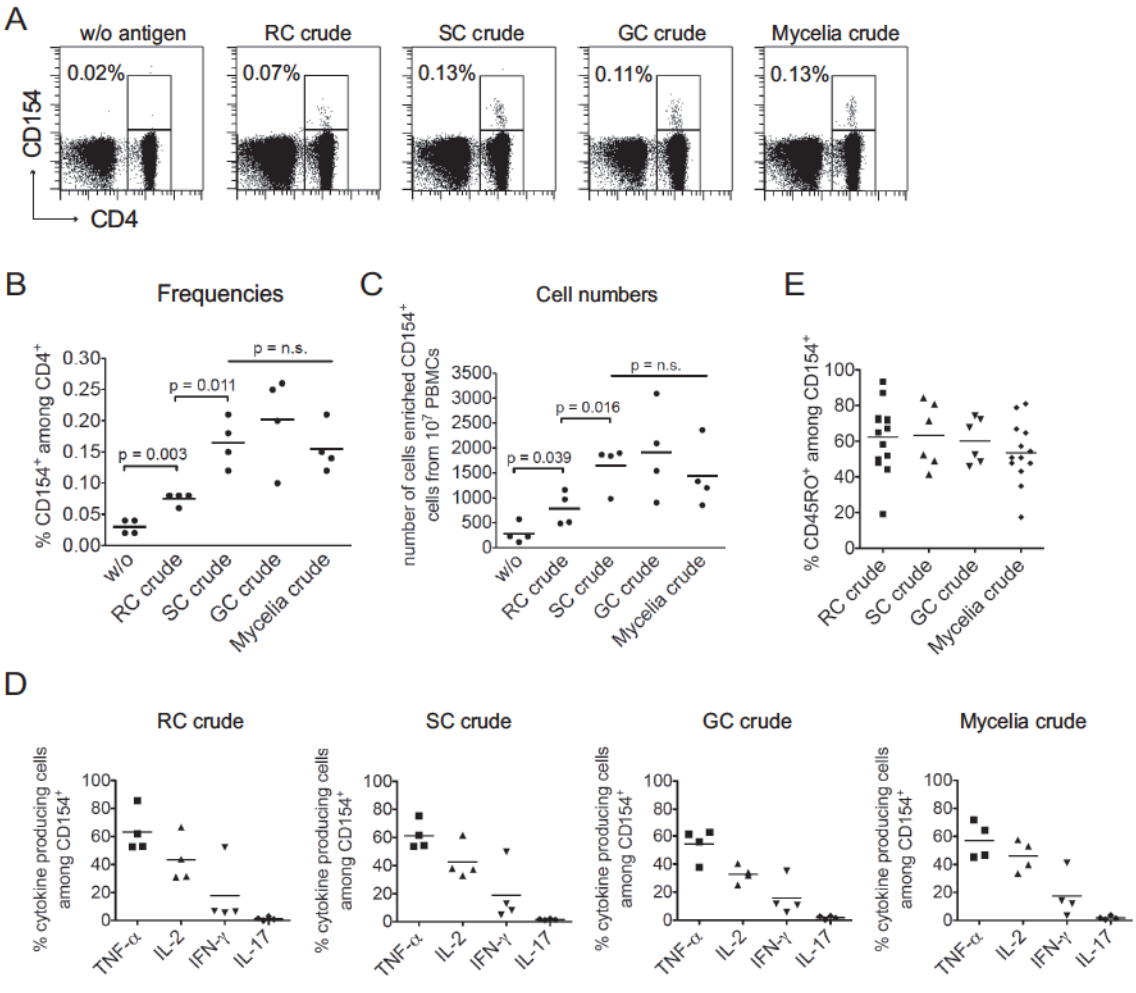
A
w/o antigen
RC crude
SC crude
GC crude
Mycelia crude
0.02%
0.07%
0.13%
0.11%
0.13%
CD154
CD4
B
Frequencies
% CD154+ among CD4+
p = 0.003
p = 0.011
p = n.s.
w/o
RC crude
SC crude
GC crude
Mycelia crude
C
Cell numbers
number of cells enriched CD154+ cells from 10^7 PBMCs
p = 0.039
p = 0.016
p = n.s.
E
% CD45RO+ among CD154+
D
RC crude
SC crude
GC crude
Mycelia crude
% cytokine producing cells among CD154+
TNF-α
IL-2
IFN-γ
IL-17

Figure 2

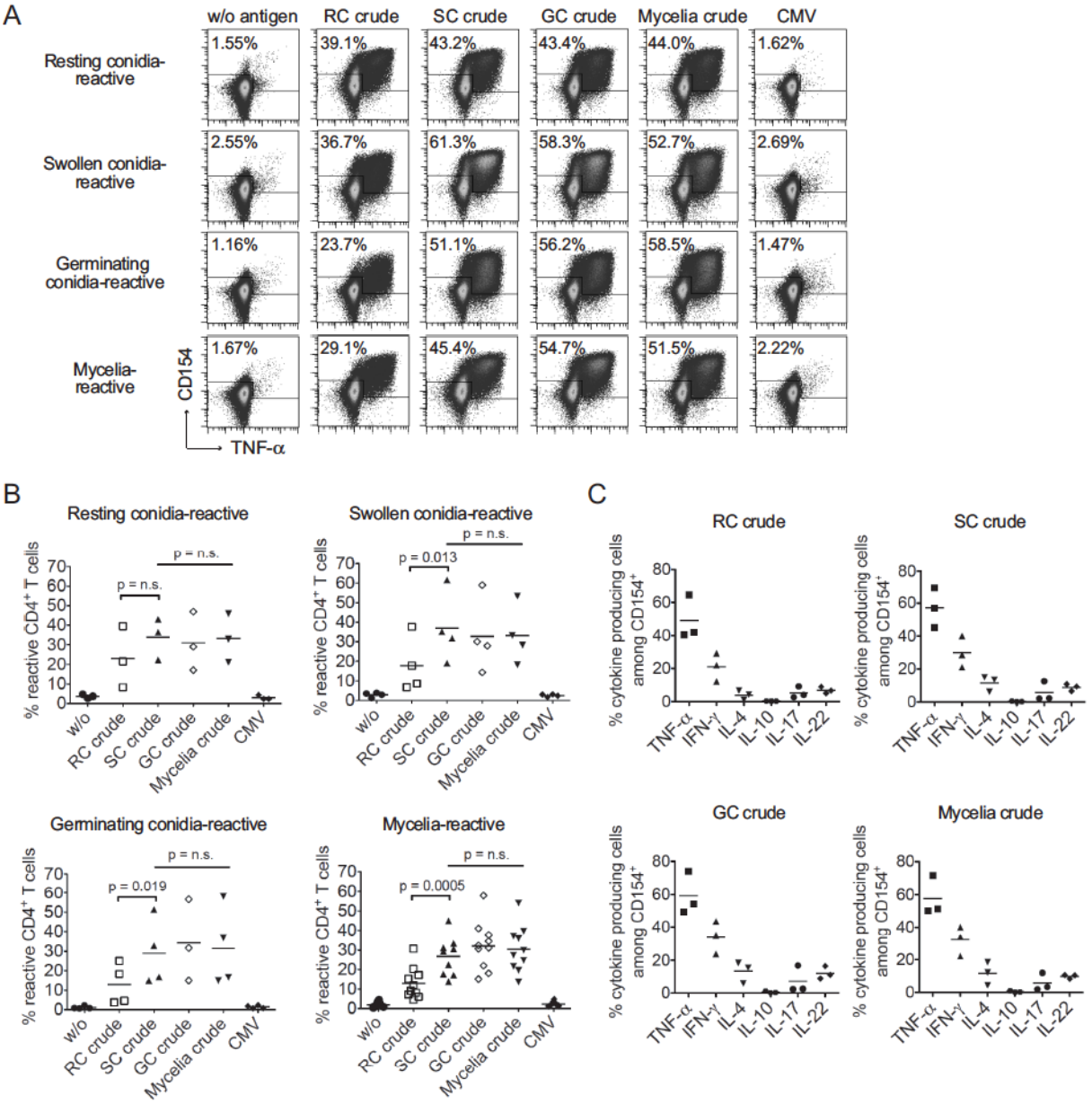
A
w/o antigen
RC crude
SC crude
GC crude
Mycelia crude
CMV
Resting conidia-reactive
Swollen conidia-reactive
Germinating conidia-reactive
Mycelia-reactive
1.55%
39.1%
43.2%
43.4%
44.0%
1.62%
2.55%
36.7%
61.3%
58.3%
52.7%
2.69%
1.16%
23.7%
51.1%
56.2%
58.5%
1.47%
1.67%
29.1%
45.4%
54.7%
51.5%
2.22%
CD154
TNF-α
B
Resting conidia-reactive
Swollen conidia-reactive
Germinating conidia-reactive
Mycelia-reactive
% reactive CD4+ T cells
p = n.s.
p = 0.013
p = 0.019
p = 0.0005
w/o
RC crude
SC crude
GC crude
Mycelia crude
CMV
C
RC crude
SC crude
GC crude
Mycelia crude
% cytokine producing cells among CD154+
TNF-α
IFN-γ
IL-4
IL-10
IL-17
IL-22

Figure 3

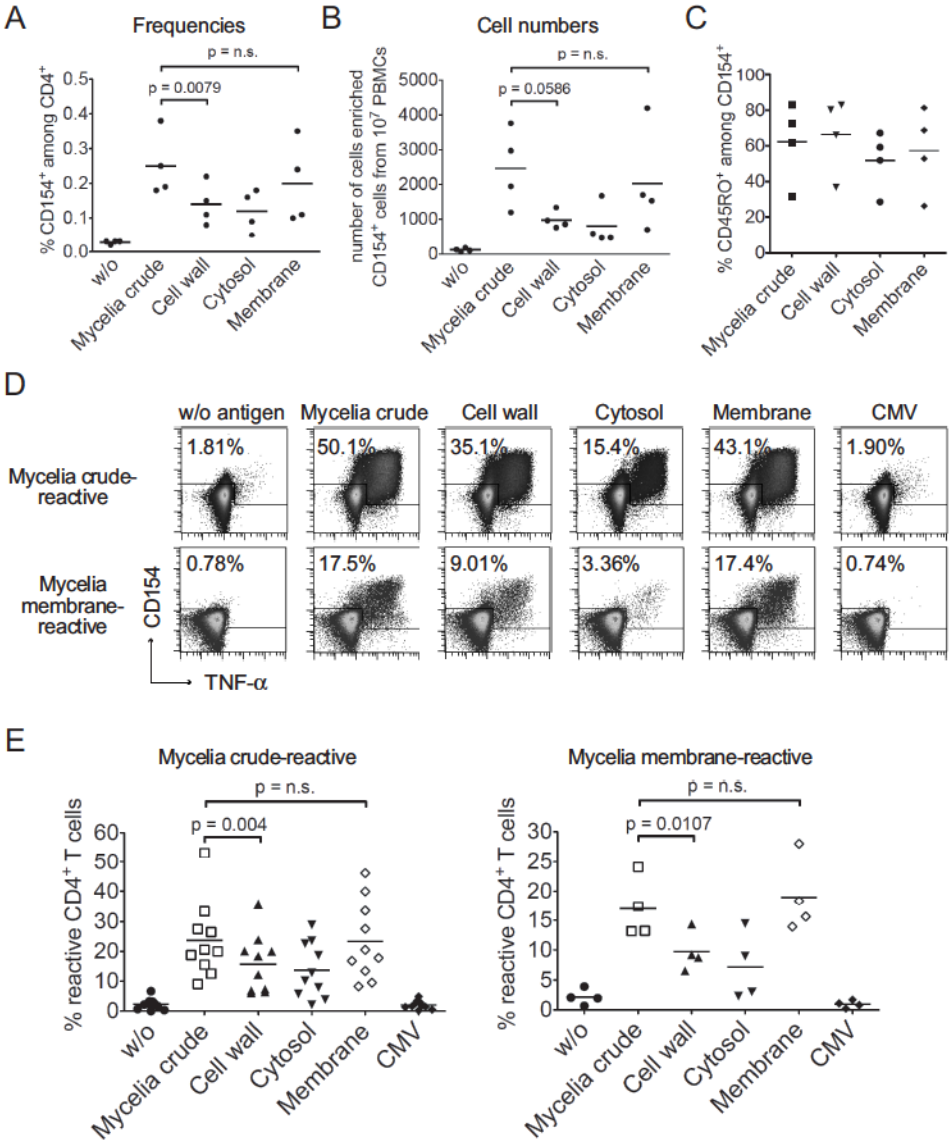
A
Frequencies
% CD154+ among CD4+
p = n.s.
p = 0.0079
w/o
Mycelia crude
Cell wall
Cytosol
Membrane
B
Cell numbers
number of cells enriched CD154+ cells from 10^7 PBMCs
p = n.s.
p = 0.0586
C
% CD45RO+ among CD154+
D
w/o antigen
Mycelia crude
Cell wall
Cytosol
Membrane
CMV
Mycelia crude-reactive
Mycelia membrane-reactive
1.81%
50.1%
35.1%
15.4%
43.1%
1.90%
0.78%
17.5%
9.01%
3.36%
17.4%
0.74%
CD154
TNF-α
E
Mycelia crude-reactive
Mycelia membrane-reactive
% reactive CD4+ T cells
p = n.s.
p = 0.004
p = 0.0107
CMV

Figure 4

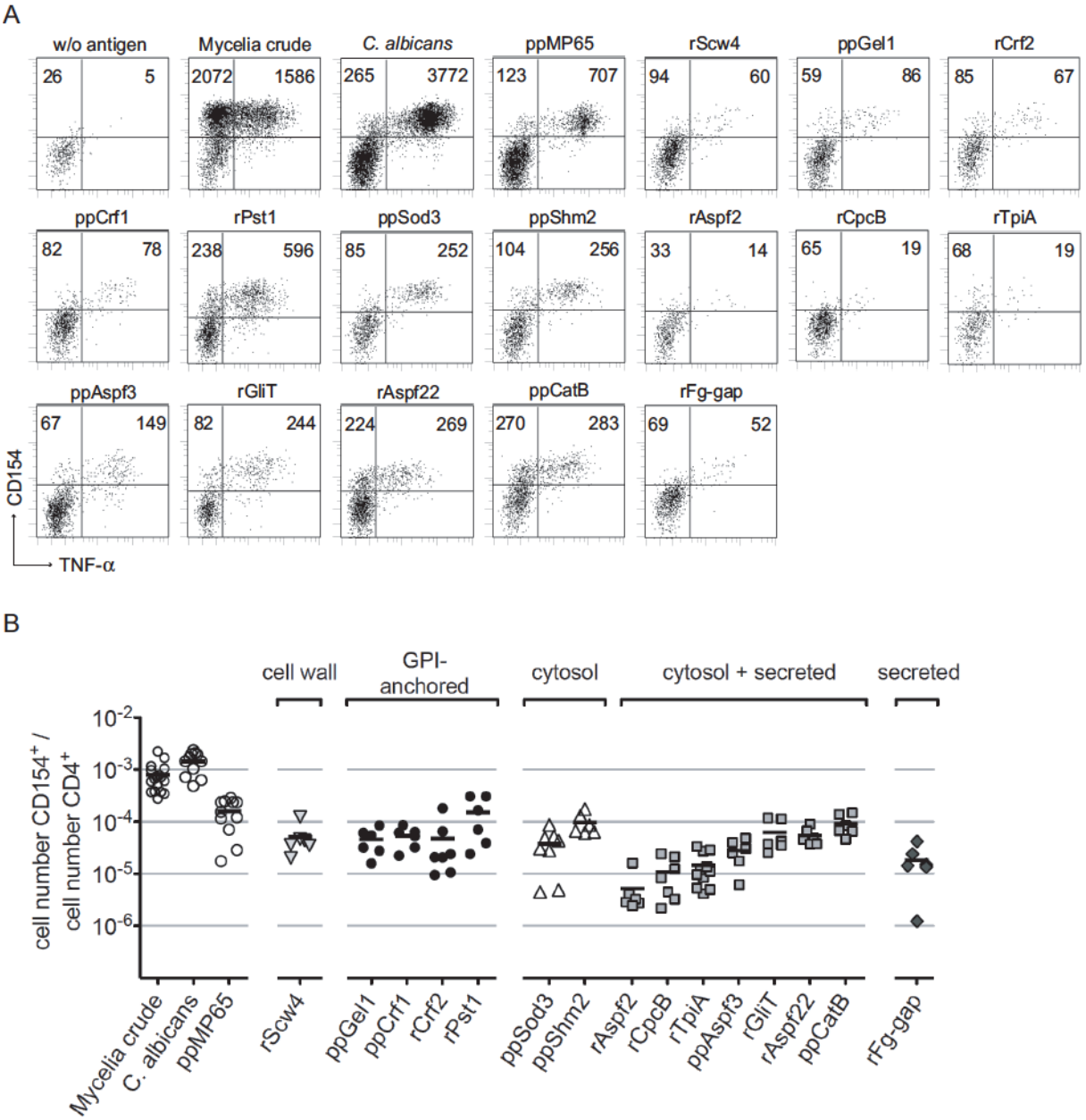
A
w/o antigen
Mycelia crude
C. albicans
ppMP65
rScw4
ppGel1
rCrf2
26
5
2072
1586
265
3772
123
707
94
60
59
86
85
67
ppCrf1
rPst1
ppSod3
ppShm2
rAspf2
rCpcB
rTpiA
82
78
238
596
85
252
104
256
33
14
65
19
68
19
ppAspf3
rGliT
rAspf22
ppCatB
rFg-gap
67
149
82
244
224
269
270
283
69
52
CD154
TNF-α
B
cell wall
GPI-anchored
cytosol
cytosol + secreted
secreted
cell number CD154+ / cell number CD4+
10-2
10-3
10-4
10-5
10-6
Mycelia crude
C. albicans
ppMP65
rScw4
ppGel1
ppCrf1
rCrf2
rPst1
ppSod3
ppShm2
rAspf2
rCpcB
rTpiA
ppAspf3
rGliT
rAspf22
ppCatB
rFg-gap

Figure 5

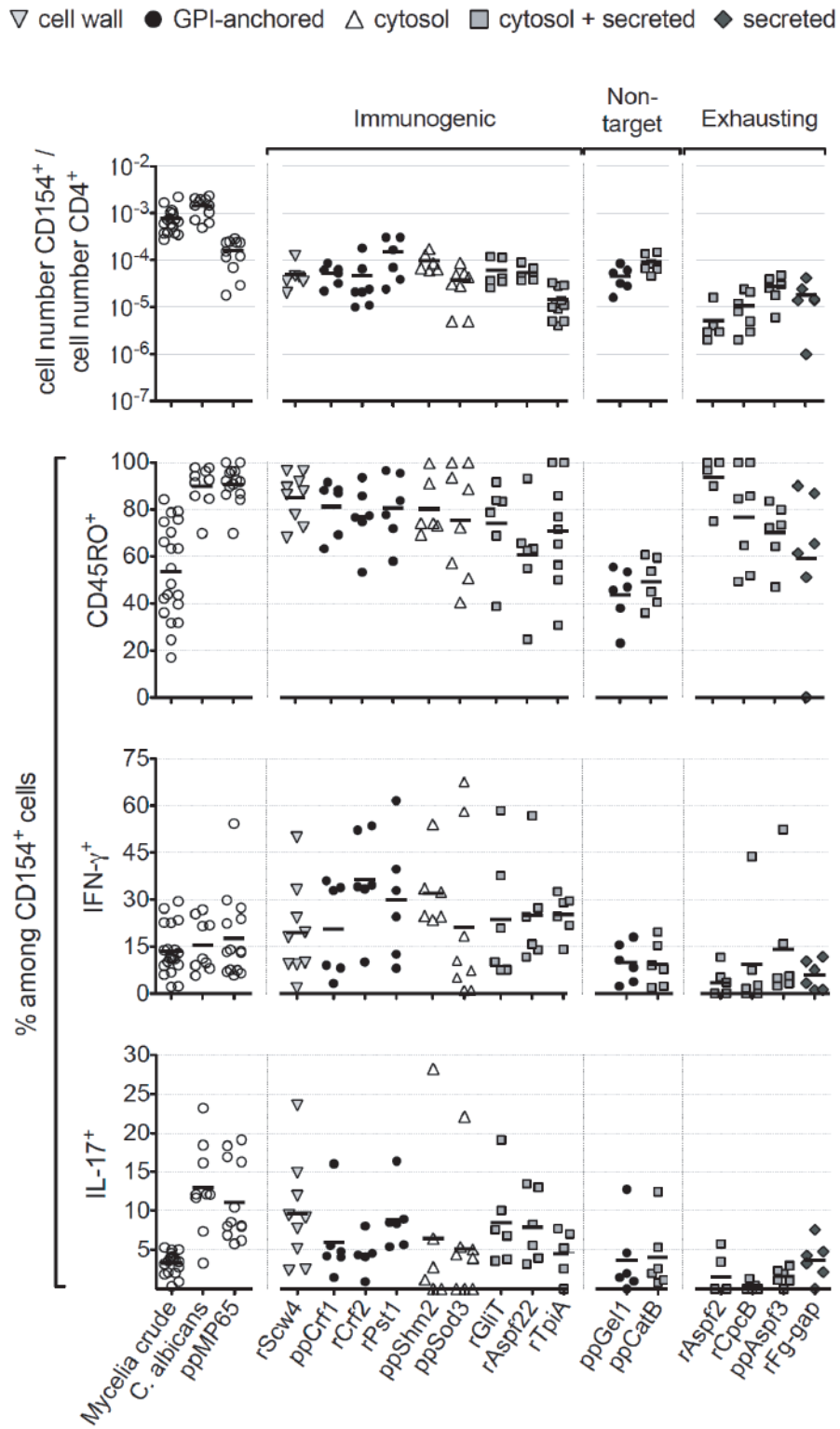
cell wall
GPI-anchored
cytosol
cytosol + secreted
secreted
Immunogenic
Non-target
Exhausting
cell number CD154+ / cell number CD4+
% among CD154+ cells
CD45RO+
IFN-γ+
IL-17+
Mycelia crude
C. albicans
ppMP65
rScw4
ppCrf1
rCrf2
rPst1
ppShm2
ppSod3
rGliT
rAspf22
rTpiA
ppGel1
ppCatB
rAspf2
rCpcB
ppAspf3
rFg-gap

Figure 6

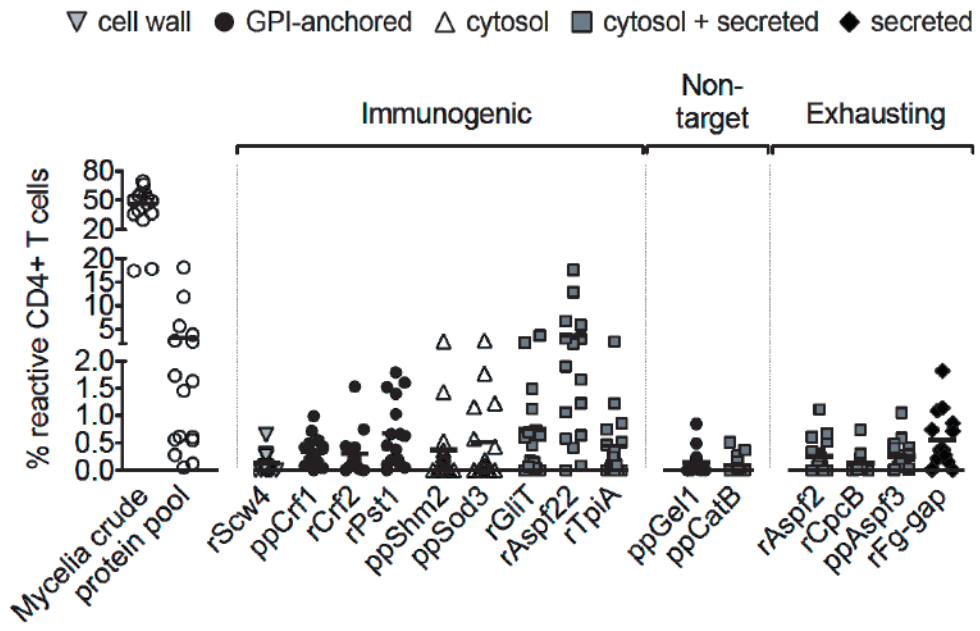
cell wall
GPI-anchored
cytosol
cytosol + secreted
secreted
Immunogenic
Non-target
Exhausting
% reactive CD4+ T cells
80
50
20
20
15
10
5
2.0
1.5
1.0
0.5
0.0
Mycelia crude protein pool
rScw4
ppCrf1
rCrf2
rPst1
ppShm2
ppSod3
rGliT
rAspf22
rTpiA
ppGel1
ppCatB
rAspf2
rCpcB
ppAspf3
rFg-gap

Figure 7

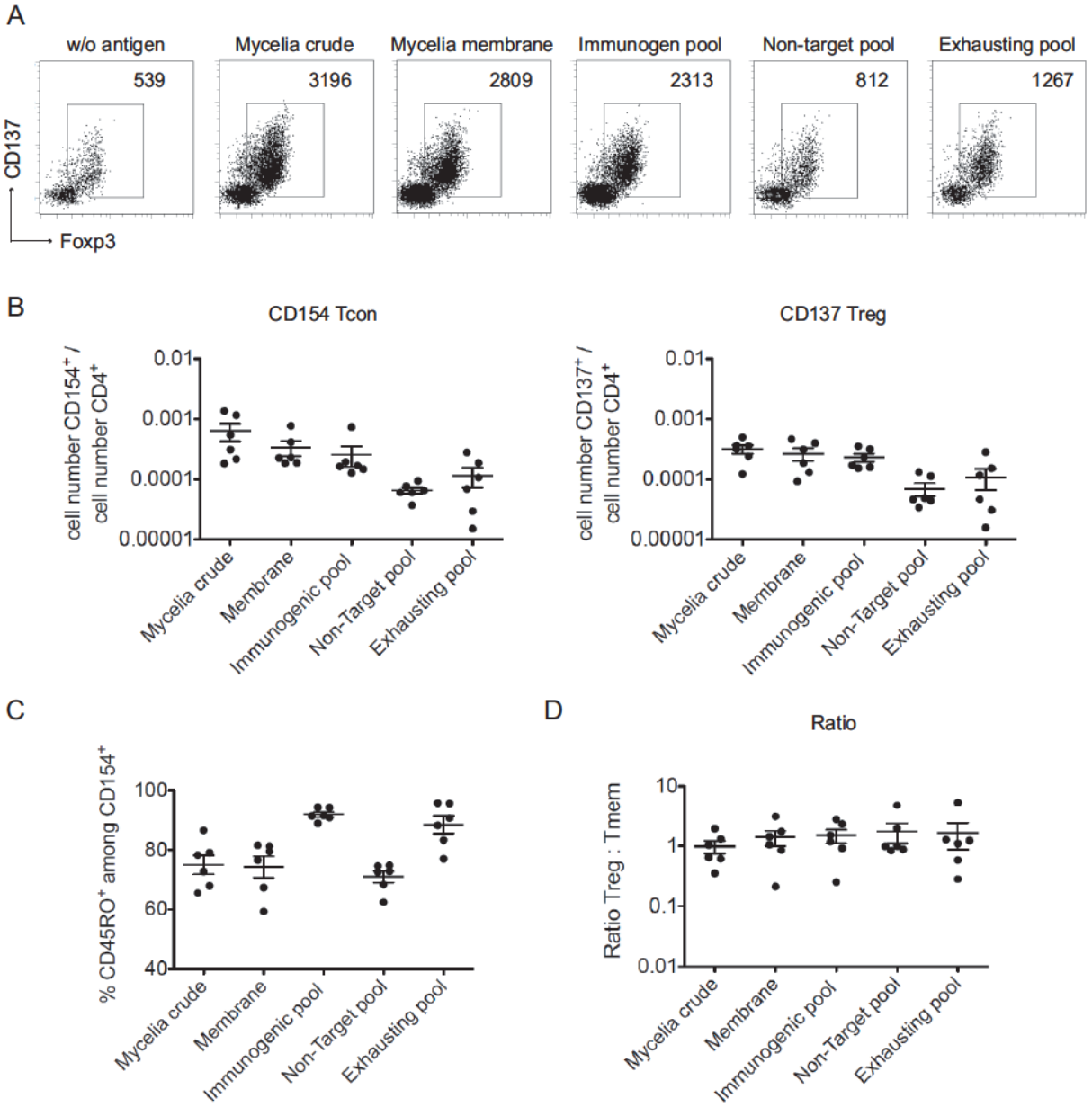
A
w/o antigen
Mycelia crude
Mycelia membrane
Immunogen pool
Non-target pool
Exhausting pool
539
3196
2809
2313
812
1267
CD137
Foxp3
B
CD154 Tcon
CD137 Treg
cell number CD154+ / cell number CD4+
cell number CD137+ / cell number CD4+
0.01
0.001
0.0001
0.00001
Mycelia crude
Membrane
Immunogenic pool
Non-Target pool
Exhausting pool
C
D
% CD45RO+ among CD154+
100
80
60
40
Ratio
Ratio Treg : Tmem
10
1
0.1
0.01

Figure S1

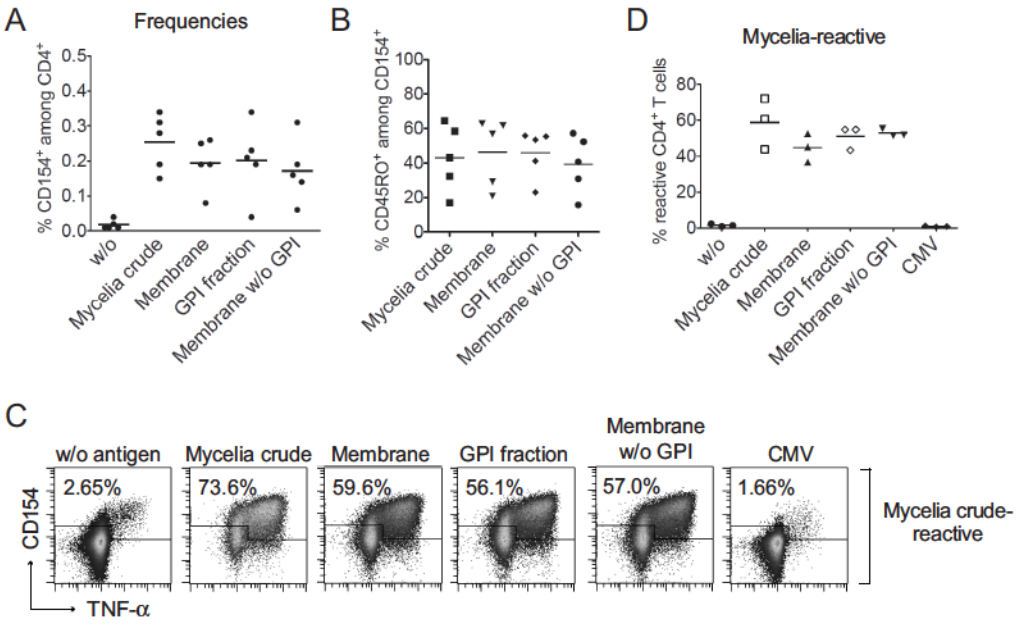

Figure S1. GPI-anchored proteins and the non-GPI membrane fraction have similar stimulatory capacities. (A) Frequencies of $CD154^+$ cells among $CD4^+$ lymphocytes following *ex vivo* stimulation with the indicated lysates. (B) $CD154^+$ cells were enriched from 1×10^7 PBMCs and analyzed for surface expression of CD45RO. Percentage of $CD45RO^+$ memory cells among $CD154^+$ cells are depicted. (C, D) Specific T cell lines were expanded from enriched crude mycelia lysate stimulated $CD154^+$ T cells, and analyzed for reactivity by antigen re-stimulation. (C) Representative dot plot examples from one donor and (D) statistical analysis.

Figure S2

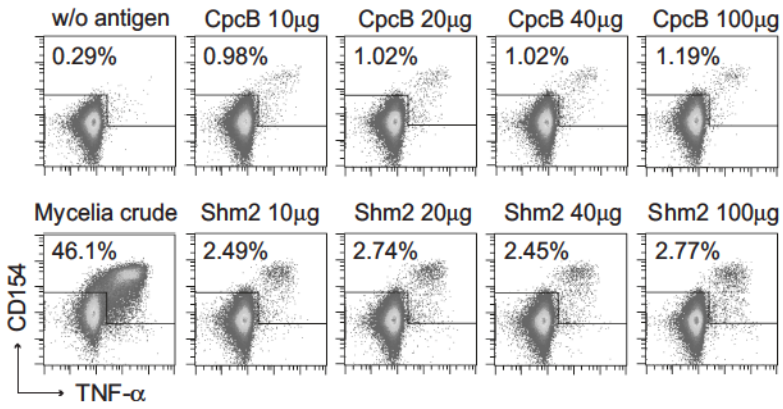

Figure S2. Titration of recombinant proteins on expanded mycelia-reactive T cell lines. Isolated crude mycelia lysate-reactive $CD154^+$ T cells were expanded for 2 weeks and re-stimulated with or without mycelia lysate, or different concentrations of recombinant CpcB and Shm2 protein. Percentages of reactive cells gated on $CD4^+$ lymphocytes are shown, as determined by CD154 and TNF-α expression.

Figure S3

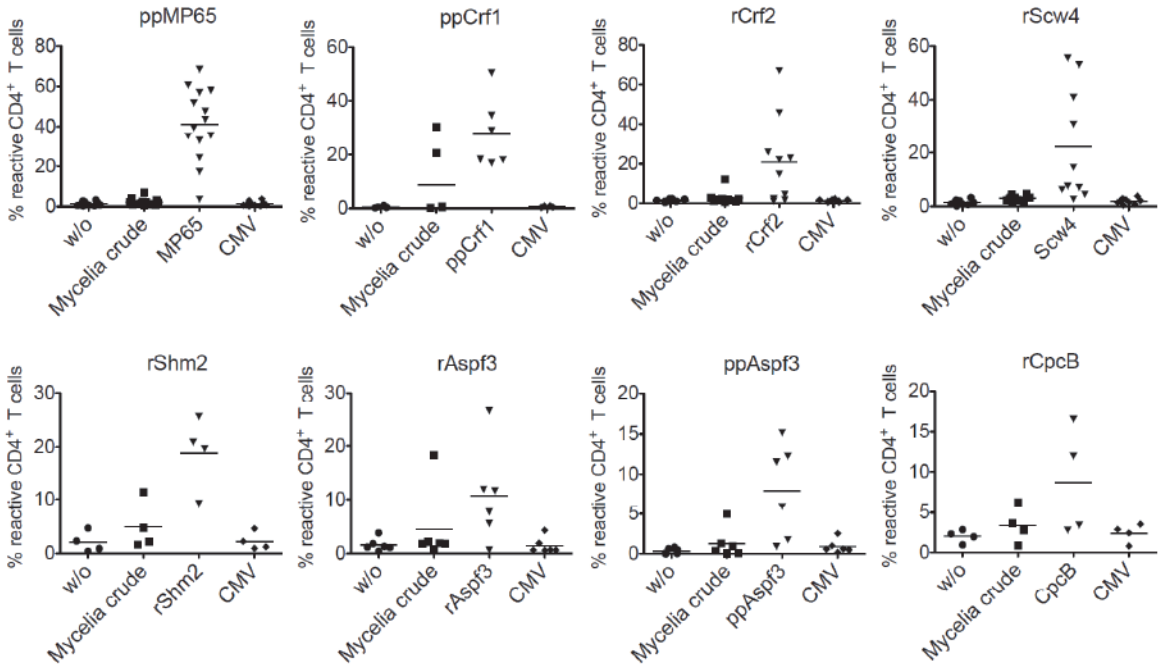

Figure S3. Specificity of single protein-reactive CD154$^+$ T cells. PBMCs were stimulated with the indicated proteins. CD154$^+$ cells were isolated, subsequently expanded for 3 weeks and tested for specificity via antigen re-stimulation. Percentage of reactive cells among CD4$^+$ lymphocytes are shown for several donors, as determined by CD154 and TNF-α expression.

Table 1. Overview of single *A. fumigatus* proteins used in this study

Protein	Locus-tag	Function	Localization	Length	Preparation	Identified in conidial proteome	Identified in mycelial proteome	Identified in secretome	Identified in patients sera immunoblots
Scw4	AFUA_6G12380	Cell wall glucanase	Cell wall	369 aa	Recombinant	n.d.	n.d.	n.d.	n.d.
Crf2	AFUA_1G16190	Cell wall glucanase	GPI-anchored	333 aa	Recombinant	n.d.	x (25)	n.d.	n.d.
Gel1	AFUA_2G01170	1,3-β-glucanosyltransferase	GPI-anchored	452 aa	Peptide pool	x (41)	x (41)	x	x (32)
Crf1/Aspf9	AFUA_1G16190	Cell wall glucanase	GPI-anchored, secreted	395 aa	Peptide pool	-	x (25)	x (56)	x (32)
Pst1	AFUA_6G10290	GPI-anchored cell wall protein	GPI-anchored	405aa (aa 18-216 expressed)	Recombinant	n.d.	n.d.	n.d.	n.d.
Sod3/Aspf6	AFUA_1G14550	Manganese superoxide dismutase	Cytosol	210 aa	Peptide pool	-	x (57)	-	x (57)
Shm2	AFUA_3G09320	Serine hydroxylmethyltransferase	Cytosol	471 aa	Peptide pool	x (41)	x (21, 41)	-	x (58)
Aspf2	AFUA_4G09580	allergen, expressed in zinc-limiting conditions	Cytosol, Secreted	310 aa	Recombinant	n.d.	x (59)	x	x (49, 60, 61)
CpcB	AFUA_4G13170	RACK1 orthologue	Cytosol, Secreted	316 aa	Recombinant	x (21, 40)	x (22)	x (56)	x (45)
TpiA	AFUA_5G13450	Triosephosphate isomerase	Cytosol, Secreted	256 aa	Recombinant	x (40)	x (22)	x (56)	x (62)
Aspf3	AFUA_6G02280	Peroxiredoxin family reductase	Cytosol, Secreted	168 aa	Peptide pool	x (41)	x (21)	-	x (62)
GliT	AFUA_6G09740	Gliotoxin oxidase	Cytosol, Secreted	334 aa	Recombinant	n.d.	x (63)	x (63)	x (45)
Aspf22/EnoA	AFUA_6G06770	Enolase	Cytosol, Secreted	438 aa	Recombinant	x (21, 40)	x (22)	x (56)	x (58)
CatB	AFUA_3G02270	Catalase B	Cytosol, Secreted	728 aa	Peptide pool	x (21)	x (22)	x (56)	x (49)
FG-GAP	AFUA_1G04130	FG-GAP repeat protein	Secreted	307 aa (aa 26-307 expressed)	Recombinant	n.d.	-	x (56)	x (49)

aa amino acid; n.d. not determined

4 Discussion

Fungal pathogens significantly contribute to morbidity and mortality in humans. However, their impact on human health and diseases has been underestimated for long time (Brown et al., 2012). Accordingly, much less is known about the T cell response against fungal pathogens in comparison for example to viruses, which much earlier have been recognized to cause fatal infections in immunocompromised patients and subsequently protective immune responses have extensively been studied. As adoptive transfer of virus-specific T cells has been demonstrated to confer protective immunity and to control established viral infections (Brestrich et al., 2009; Feuchtinger et al., 2006; Mackinnon et al., 2008; Moosmann et al., 2010), similar approaches could be beneficial to fight the devastating fungal infections in immune-suppressed patients (Beck et al., 2006; Gaundar et al., 2012; Perruccio et al., 2005; Tramsen et al., 2009; Tramsen et al., 2013). Also, the development of anti-fungal vaccines might be an option to efficiently protect immunocompromised patients from fungal infections (Spellberg, 2011; Stevens et al., 2011). However, basic knowledge about fungus-specific T cells is missing that would be essential to define protective anti-fungal T cell responses and to consider infusion of fungus-specific T cells as a treatment option.

Besides being a major cause of infection-related mortality in immune-suppressed patients, environmental airborne fungi are increasingly associated with allergic hypersensitivity diseases. Especially patients with severe asthma commonly show sensitization to fungal antigens (Agarwal, 2011; Agarwal and Gupta, 2011). In addition, susceptible patients with asthma and cystic fibrosis frequently suffer from ABPA. It is likely, that the T cell response differs under these divers pathologic situations, as invasive fungal infections result from an insufficient immune response against the fungal pathogens, whereas allergic disorders are the consequence of an aberrant immune response due to sensitization to fungal antigens. To define a role for T cells in anti-fungal immunity under these different pathologic conditions, it demands comprehensive information about anti-fungal T cell responses in humans.

4.1 Direct, high-resolution analysis of the total anti-fungal human $CD4^+$ T cell response

Most of the current data about fungus-specific T cells result from experiments with animal models. However, a variety of different models for invasive, disseminated, allergic, mucosal and superficial fungal disease have been described with several variables regarding the strain of mouse or other animal species used, the dose and morphotype of the fungal antigens, the route of infection, or usage and kind of immune-suppression and therapy (Capilla et al., 2007; Kamei, 2001). These methodological differences among the different models make a direct comparison of results from individual studies difficult (Sheppard et al., 2006). In addition, as for other diseases, it is delicate to draw direct conclusions from results of animal studies on humans, even if the animal model mimics the human disease as best as possible. For example, models with sterile housed animals do not consider the effect of preexisting immunological memory acquired during exposure to the multitude of different antigens in an individuals life. Thus, analyses of antigen-specific T cells in humans need to be obtained. The comparison of anti-fungal T cell immunity in healthy donors, as well as the relevant patient groups is essential to define correlates of disease progression and protection against fungus-mediated diseases. In detail, knowledge about the frequencies, antigen-specificity, phenotype and function of fungus-specific T cells is only fragmentary or lacking all together.

A. fumigatus and *C. albicans* are two of the major opportunistic fungal pathogens in humans, but so far only few studies have analyzed *A. fumigatus* and *C. albicans* specific T cells in human peripheral blood (Acosta-Rodriguez et al., 2007b; Beck et al., 2006; Bozza et al., 2009; Chai et al., 2010; Chaudhary et al., 2010; Hebart et al., 2002; Jolink et al., 2013; Liu et al., 2009; Stuehler et al., 2011; Zhou et al., 2008; Zielinski et al., 2012). In addition, many of these studies measured the fungus-specific T cell response by indirect methods, such as secretion of cytokines into the supernatant or proliferation of the activated T cells. A problem of such approaches is that one cannot draw conclusions on the number of antigen-specific cells at the time point of stimulation, since differences in the amount of cytokine production or proliferation between the individual cells are not considered. Furthermore, when stimulating bulk cell populations, such as whole PBMC, also other cells may secrete the measured cytokine, and proliferation due to bystander activation cannot be excluded. Other

studies measured human fungus-specific T cells by the detection of single cytokines using ELIspot assay or flow-cytometry. However, this restricts the analysis to only one or few cytokines whereas specific T cells producing different or no cytokines are being missed. Also, ELIspot assays do not provide additional information about the individual antigen-specific T cells, like the expression of phenotypic markers or co-secretion of several cytokines, as it applies for flow-cytometric measurements. Thus, the current data about the frequency and functions of fungus-specific T cells in humans are only incomplete and/or largely imprecise. Although MHC-multimers would enable a direct quantification and characterization of fungus-specific T cell responses, they are currently not applicable to fungal antigens, since no immune-dominant proteins or epitopes are known. In addition, also the MHC-multimer technology would again only give a very restricted view on those T cells with specificity for the pre-selected epitopes, which most likely does not reflect the entire T cell response against such complex organisms like fungal pathogens.

To gain a more comprehensive picture about fungus-specific T cell responses in humans, a flow-cytometric assay based on the expression of CD154 was developed (antigen-reactive T cell enrichment, ARTE) that enabled the detection of the total pool of specific $CD4^+$ T cells, independent of MHC-allele variation, defined antigenic epitopes or restriction to certain phenotypic or functional subsets (Bacher et al., 2013; manuscript 2). CD154 has previously been shown to be a highly specific marker that is up-regulated on all antigen-specific $CD4^+$ T cells following short *in vitro* stimulation (Chattopadhyay et al., 2005; Frentsch et al., 2005; Kirchhoff et al., 2007). The specificity of CD154 induction following stimulation with crude fungal lysates was confirmed in this study, since the induction of CD154 by fungal lysates was HLA-DR dependent and required processing by APCs. In addition, fungus-specific T cell lines or single cell clones with high reactivity could be generated following expansion of enriched $CD154^+$ T cells (Bacher et al., 2013; manuscript 2; Bacher et al.; manuscript 6, *submitted*). The magnetic pre-enrichment of the rare $CD154^+$ T cells enabled to rapidly analyze sufficient numbers of target cells to discriminate even small subpopulations that were previously undetectable, *e.g.* naive or rare cytokine-producing cells. Indeed, the analysis of the total pool of fungus-specific T cells revealed, that restriction of the analysis to only the dominant lineage defining cytokines (*e.g.* IFN-γ, IL-4, IL-17) largely underestimates the actual frequencies of fungus-reactive $CD4^+$ T cells, since the production of effector cytokines among all $CD154^+$ T cells was only low (Bacher et al., manuscript 6, *submitted;* Bacher et al., 2013; manuscript 2).

Only recently it has been identified that the combination of CD154 expression with another activation marker, CD137, enables the discrimination between antigen-specific human Tcon and Treg (Schoenbrunn et al., 2012). The applicability of this marker combination to also identify pathogen-specific Treg and Tcon was demonstrated, since *A. fumigatus*- and *C. albicans*-specific $CD137^+CD154^-$ Th cells displayed hallmarks of Treg, such as expression of characteristic markers ($CD25^{high}$, $CD127^-$, $Foxp3^+$, $Helios^+$), demethylated TSDR, *in vitro* suppressive function and absence of effector cytokine expression. In contrast, $CD154^+CD137^-$ Th cells matched with the properties of conventional $CD4^+$ T cells (Bacher et al., 2013; manuscript 3). By combining both markers with the pre-enrichment strategy, the flow-cytometric assay developed in this study allows for the first time the direct *ex vivo* identification and characterization of the unrestricted $CD4^+$ Tcon and Treg pool specific for any antigen of interest on a single cell level, with the same sensitivity as it was demonstrated for multimer-based enrichment methods (~ 1 cell within 10^5-10^6, depending on the number of input cells) (Bacher et al., 2013; manuscript 3; Bacher and Scheffold, 2013; manuscript 1; Bacher et al., 2013; manuscript 2)

The high sensitivity of the antigen-reactive enrichment assay and the possibility to analyze the entire fungus-specific $CD4^+$ T cell compartment revealed profound differences in the human $CD4^+$ T cell response against *A. fumigatus* and *C. albicans*, with regard to frequency of the specific T cells, effector functions, phenotype, as well as the contribution of specific Treg to the anti-fungal immune response. In line with the constitutive exposure to fungal antigens, low frequencies of fungus-reactive T cells could be detected in all healthy donors (Bacher et al., 2013; manuscript 3; Bacher et al., manuscript 6, *submitted*; Bacher et al., manuscript 5, *in preparation*). However, *C. albicans* induces a clear memory $CD4^+$ T cell response with a considerable proportion of Th17 cells, which is accompanied by a parallel expansion of Treg. In contrast, the overall T cell response against *A. fumigatus* seems to be more tolerogenic, since a large proportion of the *A. fumigatus*-specific $CD4^+$ T cells is still in a naive state, and exposure to *A. fumigatus* preferentially expands Treg (Bacher et al., 2013; manuscript 3). In addition, a significant proportion of the *A. fumigatus*-reactive memory response was induced by cross-reactivity to *C. albicans* antigens, leading to the selective induction of Th17 cells in the *A. fumigatus* immune response (Bacher et al., manuscript 4, *in preparation*). These fundamental differences in the T cell response against *A. fumigatus* and *C. albicans* were surprising, since the human immune system is continuously exposed to both fungi. However, *C. albicans* is a dimorphic yeast and commensal mainly residing on the mucosal surface of the gastrointestinal tract. Interaction with the immune system mainly occurs upon *C. albicans*

penetration of the usually protective mucous layer (Cheng et al., 2012; Naglik et al., 2011). In contrast, *A. fumigatus* is an environmental fungal organism that is primary acquired to the lung by inhaling airborne spores. Thus, *A. fumigatus* and *C. albicans* largely differ in several parameters that might be critically for the outcome of a protective immune response, including the expression of different pathogen-associated molecular patterns (PAMPs), the timing, dosage and kind of antigen exposure, as well as the site of interaction with the human immune system.

4.2 Expanded fungus-specific Treg in human peripheral blood

All humans are constitutively exposed to several environmental antigens, such as allergens, food, commensal bacteria and fungi. Fungi represent a challenge for the immune system, as immune ignorance of fungal antigens will lead to uncontrolled fungal growth and invasive fungal diseases, as best illustrated by the devastating fungal infections in immune-suppressed patients. On the other hand overreacting or chronic immune reactions against these daily encountered antigens may result in collateral tissue damage and allergic disorders. Thus, immunity to fungi demands appropriate protective immune responses but also efficient control mechanisms to prevent tissue damage due to overreacting or chronic immune reactions. In addition, the anatomical site where the immune system is confronted with the fungal antigen can vary widely, since airborne fungal spores are inhaled into the lung, whereas other fungi live as commensals in the human gastrointestinal tract or on the skin. How the immune system copes with this daily challenge against the different fungal antigens is so far only poorly understood.

Treg are well recognized in their capacity to control immune responses against self-antigens (Sakaguchi et al., 2010), and have also been implicated to maintain immune homeostasis against foreign antigens at mucosal sites (Belkaid and Rouse, 2005; Bilate and Lafaille, 2012; Curotto de Lafaille et al., 2008; Curotto de Lafaille and Lafaille, 2009; Josefowicz et al., 2012). However, the antigen-specificity of the Treg repertoire remained largely undefined. Especially in humans the direct detection of pathogen-specific Treg failed so far, due to the lack of appropriate methods for their identification. It is of particular interest that both, *A. fumigatus* and *C. albicans,* elicit a strong expansion of specific Treg in adult healthy donors, compared to cord blood (Bacher et al., 2013; manuscript 3). Also colonization of mice with commensal bacterial species, in particular *Clostridium* species and *Bacteroides*

fragilis, has been demonstrated to promote Treg accumulation in the intestinal mucosa (Atarashi et al., 2011; Round and Mazmanian, 2010). This clearly suggests the involvement of Treg in mediating homeostasis to gastrointestinal commensals and at least some of these colonic Treg have been demonstrated to express TCRs specific for colonizing bacteria (Lathrop et al., 2011). In addition, in several studies using mouse models of chronic infection with parasites, bacteria and viruses, an increased frequency of Treg at inflammatory sites has been reported (Belkaid and Tarbell, 2009; Shafiani et al., 2013), suggesting that Treg co-expand together with Tcon during an immune response and thus are part of the physiological T cell response against foreign antigens. However in the majority of these studies the specificity of the recruited Treg has not been demonstrated, leaving open the possibility that Treg accumulation during infection occurs due to bystander activation.

The expansion of *C. albicans*-specific Treg in humans occurred along with a strong effector T cell response (Bacher et al., 2013; manuscript 3). In fact, the analysis of the total *C. albicans*-specific $CD4^+$ T cell repertoire revealed that the naive T cell pool was largely depleted from *C. albicans*-reactive T cells compared to cord blood, whereas *C. albicans*-specific memory cells were strongly expanded and clearly exceeded the number of specific Treg. This supports the hypothesis that the occasional interaction of *C. albicans* with the immune system obviously leads to a rather strong immune response that is controlled by a parallel expansion of Treg to avoid detrimental consequences to the host. Nevertheless, the frequency of *C. albicans*-specific Tcon and Treg in human gut tissues remains to be explored.

In contrast to *C. albicans*, *A. fumigatus* elicited only a minimal expanded memory response in healthy donors, whereas a large proportion of the specific Tcon repertoire was still in a naive state. However, *A. fumigatus*-specific Treg were equal or in some donors even exceeded the number of specific naive and memory Tcon in healthy donors. Furthermore, *A. fumigatus* Treg strongly suppressed the Tcon response in *in vitro* assays (Bacher et al., 2013; manuscript 3). Therefore it seems that the continuous inhalation of airborne *A. fumigatus* antigens preferentially generates a Treg response, which might play an important role in the mediation of airway tolerance. In line with this idea, a recent study highlighted that lung resident macrophages of mice have a unique capacity to promote Treg responses against airborne antigens (Soroosh et al., 2013). Furthermore, tetramer enrichment approaches have identified human Treg specific for epitopes of birch or alder allergens in humans (Palomares et al., 2011; Wambre et al., 2012). In summary, these data point out that the lung might be a site for the preferential generation and/or induction of Treg responses that provide airway tolerance to inhaled antigens. Also our own preliminary data further indicate that the $CD4^+$ T cell

responses of healthy donors against several allergen lysates (*e.g.* birch pollen, ryegrass pollen, house dust mite) display the same pattern as *A. fumigatus*-specific T cells with a strong expansion of allergen-specific Treg, while leaving the majority of specific Tcon in a naive state (Bacher and Scheffold, unpublished data).

Two main types of Treg have been identified, that both contribute to the maintenance of immune tolerance: tTreg that develop in the thymus, as well as pTreg that develop outside the thymus under a variety of conditions, including infections, chronic inflammation and antigen presentation in absence of inflammation (sub-immunogenic conditions) (Bilate and Lafaille, 2012). The identified fungus-specific Treg displayed hallmarks of tTreg, *e.g.* expression of Helios, demethylated TSDR, lack of effector cytokine expression and highly suppressive capacity in *in vitro* assays (Bacher et al., 2013; manuscript 3). Although the current paradigm is that the TCR repertoire of tTreg is biased towards the recognition of self-antigens (Andersson et al., 2007), the tTreg repertoire has been demonstrated to be similar complex as the Tcon repertoire (Fazilleau et al., 2007; Kasow et al., 2004; Pacholczyk et al., 2006). Using tetramer enrichment approaches, tTreg specific for foreign epitopes have been detected in the naive repertoire of mice (Moon et al., 2011). A recent study in mice further demonstrated that tTreg contribute to tolerance to commensal bacteria (Cebula et al., 2013). These results are consistent with the idea, that tTreg can also recognize foreign antigens. Nevertheless, due to the lack of distinctive markers, it remains unclear whether the fungus-specific Treg arise from the tTreg or pTreg compartment, or whether both populations contribute to the mediation of tolerance against constantly encountered fungal antigens. Also in a recently described mouse model of colitis it has been demonstrated, that both tTreg and pTreg were required for protection from disease, probably by expanding the diversity of recognized T cell targets (Haribhai et al., 2011).

The presence of high frequencies of fungus-specific Treg in peripheral blood of healthy human donors has several important clinical implications. Especially patients with CF, who are characterized by an impaired mucociliary clearance, frequently suffer from *Aspergillus* driven allergies, which can result in severe ABPA. However, the susceptibility of some of these patients to develop allergy towards *Aspergillus* antigens, whereas others do not, are currently not well understood. Indeed, a strongly altered cytokine profile of *A. fumigatus*-specific memory T cells towards a Th2 phenotype was observed in half of the analyzed CF patients in this study (Bacher et al., 2013; manuscript 3). Strikingly, those patients with a Th2 biased phenotype, displayed a strongly reduced Treg/Tmem ratio, compared to non-Th2

patients. This suggests, that the balance between Treg and Tmem might be a decisive parameter in the development of allergy. In this regard it will be important to see, whether the reduced Treg/Tmem ratio might have a predictive value for allergy development against *A. fumigatus* antigens. The observed Treg/Tmem ratio did not result from reduced numbers of *A. fumigatus* specific Treg, but an increased number of specific Th2-type memory cells, compared to non-Th2 CF patients. Thus strategies, to enhance the number of specific Treg, might be a treatment option for CF and also otherwise allergic patients. However, as CF patients do not in general suffer from allergy against other harmless antigens (*i.e.* pollens, house dust mite, *etc.*), but selectively develop allergies against *Aspergillus*, an overall Treg defect in these patients seems unlikely. These data rather suggest, that the altered Treg/Tmem ratio is the result of an increased fungal load in the patients, maybe due to reduced phagocyte function (Donnelly and Barnes, 2012), which allows conventional T cells to escape from Treg mediated control. However, also the functional capacities of the specific Treg in CF patients still remain to be explored.

In contrast to their assumably protective function in healthy donors, the generation of a fungus-specific Treg response in immunocompromised fungus infected patients might interfere with the development of a protective anti-fungal immune response. Although a clear increase of fungus-specific $CD4^+$ Tcon frequencies was observed in fungus infected patients, the absolute numbers of reactive cells did not exceed the range of healthy donors, due to low overall counts of $CD4^+$ T cells in these patients (Bacher et al., manuscript 5, *in preparation*). As the presence of fungus-specific Treg in these strongly immune-suppressed patients has not been demonstrated so far, it will be important to determine whether fungus-specific Treg are involved in dampening the fungus-specific immune response. It has been shown previously that *A. fumigatus* spores may evade innate immune recognition by an immunological inert rodlet layer (Aimanianda et al., 2009; Bruns et al., 2010), as well as by binding of complement regulators (Behnsen et al., 2008). In this regard, the preferential activation of Treg cells by *A. fumigatus* antigens could represent a further evading strategy, which might lead to enhanced survival and persistence of the fungus. It is particular of interest that the *A. fumigatus* proteins that have been identified in this study to elicit a strong memory Tcon response in healthy donors are also the main drivers of specific Treg expansion (Bacher et al., manuscript 6, *submitted*). This, counter-regulation of the *A. fumigatus* T cell response might represent a barrier for vaccine success and depletion of Treg could be a promising strategy to elicit full anti-fungal T cell responses for immunotherapeutic approaches.

4.3 Influence of T cell cross-reactivity on anti-fungal immune responses

Upon antigen recognition, antigen-specific naive T cells undergo clonal expansion and differentiate into effector and memory cells, which provide protection upon re-exposure to the pathogen (Jenkins et al., 2001). Hence, the antigen-specific memory repertoire of an individual represents a collection of expanded T cell clones, selected within the multitude of different immune responses during the individuals´ life (Geiger et al., 2009). Although the T cell receptor repertoire is highly divers, it contains cross-reactive specificities, since the degeneracy of the T cell receptor allows recognition of similar peptides by a single TCR (Welsh and Selin, 2002). Thus, the memory T cell repertoire created in response to one antigen can influence the immune response to another unrelated antigen by T cell cross-recognition of similar or identical peptides (Selin et al., 2006; Welsh et al., 2010; Welsh and Selin, 2002).

T cell cross-reactivity of $CD8^+$ T cells seems to be a relatively common event between different viruses in murine model systems, as well as in humans (Acierno et al., 2003; Brehm et al., 2002; Clute et al., 2005; Kim et al., 2005; Nilges et al., 2003; Urbani et al., 2005; Wedemeyer et al., 2001; Welsh et al., 2010). Furthermore cross-reactive T cells have been described between viral and bacterial (Hohn et al., 2003), allogeneic (Brehm et al., 2003; Burrows et al., 1994; D'Orsogna et al., 2010) and even self-antigens (McCoy et al., 2006; Sospedra et al., 2005; Wucherpfennig and Strominger, 1995). Recent data have also suggested the existence of cross-reactive immunity between fungi. Fungal cell wall structures have been shown to confer protective immunity in mouse models, since vaccination with heat-killed *Saccharomyces* protected mice against lethal challenge with different fungi, for instance *Aspergillus, Candida* or *Coccidioides* species (Capilla et al., 2009; Stevens et al., 2011). However, whether this cross-protection is mediated by cross-reactive T cells or antibodies or whether it reflects a less specific mechanism, *i.e.* activation of innate immune cells during infection, has not been explored. In other recent studies, the generation of a $CD4^+$ TCR transgenic mouse with specificity against a shared epitope of four related dimorphic fungi (Wüthrich et al., 2011), as well as the identification of a cross-reactive T cell epitope of *A. fumigatus* and *C. albicans* (Stuehler et al., 2011) have been reported. Thus, it seems obvious, that also distantly related fungal species containing thousands of different proteins

might share highly conserved protein sequences, which can trigger cross-reactive T cell responses between fungal pathogens.

Interestingly, the current study revealed that the T cell cross-reactivity between only distantly related fungi, *i.e.* Mucorales and *Aspergillus* spp. is only low, whereas T cells reactive to *Scedosporia* spp. and *Fusaria* spp. contained a significant fraction of cells cross-reactive to *Aspergillus* spp. In addition, a strong T cell cross-reactivity between different *Aspergillus* species was identified (Bacher et al., manuscript 5, *in preparation*). These data suggest, that the level of T cell cross-reactivity reflects at least to a certain extent the actual overlap of homologous or similar antigenic epitopes and thus the developmental relationship between fungal species. However, one of the most pronounced T cell cross-reactivity was identified between the only distantly related fungal pathogens *A. fumigatus* and *C. albicans*. Intriguingly, in this case, the observed T cell cross-reactivity did not simply reflect the overlap between the *A. fumigatus* and *C. albicans* specific T cell repertoire, but rather represents the selective expansion of cross-reactive cells, since the naive *A. fumigatus*-specific T cell pool was largely depleted from cross-reacting T cells. Furthermore, the cross-reactive cells displayed phenotypical and functional characteristics of *C. albicans*-reactive T cells (Bacher et al., manuscript 4, *in preparation*). Hence, the selective expansion and the high prevalence of the cross-reactive cells might be due to the constant encounter and thus permanent triggering via *C. albicans* antigens. It will be important to further determine the protein specificity of the cross-reactive *versus A. fumigatus*-only reactive memory T cells to gain insights, whether different proteins of *A. fumigatus* are targeted in the lung and to define a role of cross-reactivity under physiological and pathological conditions.

C. albicans is a commensal on the human skin and a common part of the intestinal microbiota. The gut microbiota is known to have a major impact the immune homeostasis of the host (Backhed et al., 2005; Macpherson and Harris, 2004). For example, in germ-free mice, the adaptive immune system is largely underdeveloped (Round and Mazmanian, 2009). Inappropriate inflammatory responses to intestinal microorganisms are hypothesized to play a major role in the development and progression of inflammatory bowel diseases in genetically susceptible hosts (Abraham and Cho, 2009). In this regard it is especially of interest, that the antigen-specific T cell response against the gut commensal *C. albicans* was indeed strongly increased in patients with CD and also displayed alterations in the production of pro-inflammatory cytokines, *i.e.* higher levels of IFN-γ, IL-17 and IL-22 (Bacher et al., manuscript 4, *in preparation*). Also against other prominent gut commensals, like *E. coli*, an

increased $CD4^+$ T cell response can be detected in CD patients, however in this case IFN-γ production is strongly reduced (Bacher and Scheffold, unpublished data; Ergin et al., 2011). These data indicate that a subpopulation of patients with CD has indeed elevated T cell reactivity against gut microbiota, which is probably due to increased permeability of the intestinal barrier. However, despite the general increase in anti-microbiota T cell responses, still a pathogen-specific modulation of the adaptive immune response takes place. Thus it will be important, to find correlates of the antigen-specific T cell responses in CD patients with the course of disease, to better define the role of microbiota-specific T cell responses in IBD.

However, the intestinal microbiota is not only known to affect local immune homeostasis, but also immuno-pathology at gut-distal sites. In particular, the induction of Th17 cells has been demonstrated to depend on the presence of certain microbiota (Atarashi et al., 2008; Ivanov et al., 2009; Ivanov et al., 2008; Wu et al., 2010). Inappropriate Th17 responses have also been linked to a number of gut-distal diseases, including autoimmunity and severe lung inflammation (Akdis et al., 2012; Bettelli et al., 2007; Cosmi et al., 2011). It is not yet clear, whether the Th17 cells that arise in presence of the commensal microbiota are also specific for commensal-derived antigens, or might also recognize other antigens at distal sites. As the intestinal microbiota is highly divers it represents an extraordinary large amount of foreign antigens. Thus, one possible mechanism of how intestinal microorganisms might influence non gut-associated pathology could be T cell cross-reactivity between gastrointestinal and gut-distal antigens. Estimations suggest that the intestinal microbiota encodes indeed 100-fold more genes than the human genome (Ley et al., 2006). Thus, T cross-reactivity driven by commensal microbiota might be a common event in shaping antigen-specific T cell responses against gut-distal antigens.

In the current study it was shown that *C. albicans* driven T cell cross-reactivity has a profound influence on the T cell response against several inhaled fungal pathogens. Remarkably, up to one third of the *A. fumigatus*-specific memory T cells in healthy donors were indeed cross-reactive to *C. albicans*. Considering further, that a large proportion of *A. fumigatus*-specific conventional T cells are still in a naive state and preferentially specific regulatory memory T cells were identified in response to *A. fumigatus* (Bacher et al., 2013; manuscript 3), these data further suggest that in healthy human donors no major conventional T cell memory formation against inhaled *A. fumigatus* antigens takes place. Also in the analysis of T cell reactivity against several single *A. fumigatus* proteins, an overall higher proportion of Th17 cells was identified for many immunogenic proteins, compared to the total T cell response against the *A. fumigatus* crude extract (Bacher et al., manuscript 6, *submitted*).

This suggests, that the T cell response against these proteins might be induced via cross-reactivity to *C. albicans*. In fact, for the IL-17 inducing proteins Scw4 and Aspf 22, orthologous proteins exist in *C. albicans* (Nisini et al., 2001; Sundstrom and Aliaga, 1994).

Another important finding of this study was that the effect of cross-reactivity was not only observed on a quantitative level, but intriguingly also leads to a qualitative modulation of the T cell response against airborne fungi by selective induction of an IL-17/IL-22 producing T cell population. Although both, IFN-γ producing Th1 cells and IL-17 producing Th17 cells have been implicated to play a role in anti-fungal immune responses, the role of Th17 cells is still controversially discussed. Patients with STAT3 mutation, who lack Th17 cells, suffer from recurrent candidiasis (McDonald, 2012), but not other fungal infections, emphasizing the importance of Th17 responses for anti-*Candida* immune defense, but questioning their role in other anti-fungal immune responses. However, in several mouse models, protective roles of Th17 cells against different fungal pathogens have been described, for instance *Cryptococcus neoformans*, *Pneumocystis carinii, Blastomyces dermatitidis* (Kleinschek et al., 2006; Rudner et al., 2007; Wüthrich et al., 2013). Contrarily, other studies suggest that Th17 cells exacerbate inflammation and induce severe tissue pathology in mouse models of pulmonary aspergillosis (Romani et al., 2008; Zelante et al., 2009; Zelante et al., 2007). The deviating protective *versus* pathological role of Th17 cells in anti-fungal immunity in these studies might simply reflect experimental differences of the infection models. In addition, especially Th17 cells have been demonstrated to strongly vary between mice and humans in terms of precursors and differentiation requirements (Annunziato et al., 2009; Annunziato et al., 2010; Annunziato and Romagnani, 2009). This makes conclusions from the role of Th17 cells in animal models on the human anti-fungal T cell response difficult. Nevertheless, also in humans inappropriate Th17 responses are associated with a variety of severe lung diseases, including severe asthma, COPD and airway hypersensitivity (Alcorn et al., 2010; Cosmi et al., 2011; Halwani et al., 2013). In particular, Th17 cells have been suggested to play a role in severe asthma with fungal sensitization and cystic fibrosis (Chan et al., 2013; Tan et al., 2011). Thus, the induction of Th17 cells into the immune response against airborne fungi by cross-reactivity to *C. albicans* might even have pathological consequences in predisposed patients. In this scenario, the *C. albicans* induced cross-reactive Th17 cells might be further expanded through increased load of airborne fungal antigens in the patients´ lung and thus contribute to the disease development and/ or chronification. It will be important to determine

the prevalence of airborne fungus-reactive Th17 cells in these particular patient groups and to find potential correlates with the course and status of the diseases.

4.4 Fungus-specific $CD4^+$ T cell response during invasive fungal infections

Invasive fungal infections (IFI) are the most devastating diseases caused by environmental fungi and result in significant mortality of the infected patients. The rapid and reliable diagnosis of IFI has been shown to be crucial for a successful treatment outcome (Chamilos et al., 2008; Cornely et al., 2011), but so far only few diagnostic and prognostic tools are available in the clinical setting. The current diagnosis of IFI is difficult, because the clinical manifestations are not generally specific for fungal infections, and the available non-invasive diagnostic tools lack sensitivity and specificity (De Pauw et al., 2008; Ostrosky-Zeichner et al., 2005). Proven IFI can only be diagnosed by the histological examination of a sample taken from the infected site (De Pauw et al., 2008). This requires highly invasive methods such as biopsies or surgical resection, which are rarely feasible for most of the critically ill patients and thus represent only the last therapeutic option after unsuccessful anti-fungal treatment. Therefore, the early diagnosis of IFI and precise definition of the fungal species directly from peripheral blood samples could dramatically improve timely and targeted anti-fungal treatment and thus the outcome of invasive fungal infections.

Measurement of antigen-specific T cells has been suggested to be a promising strategy for the diagnosis of other infectious diseases, *e.g.* tuberculosis or lyme borreliosis (Forsberg et al., 1995; Harari et al., 2011; Lalvani et al., 2001; Meyer et al., 2000). Using $CD154^+$ expression as a read-out, low frequencies of reactive $CD4^+$ T cells against various opportunistic fungal pathogens were detected in all healthy donors (Bacher et al., manuscript 6, *submitted;* Bacher et al., manuscript 5, *in preparation*). However, the frequency ranges in healthy donors were quite conserved at a low level, indicating that despite chronic exposure to fungal pathogens, the T cell reactivity in healthy individuals is rather low. In contrast, strongly increased frequencies of fungus-reactive $CD4^+$ T cells were detected in patients with invasive fungal infections (Bacher et al., manuscript 5, *in preparation*). Importantly, the increased frequencies were only present during acute fungal infection, and rapidly declined following removal of the fungus by surgery. This underlines that the quantification of fungus-specific T cells represents a promising strategy for the diagnosis of IFI. The measurement of fungus-specific $CD4^+$ T cells was possible despite massive disease-associated alterations in the composition

of the patients PBMCs, demonstrating the robustness and sensitivity of the assay and the high potential to advance IFI diagnosis.

However, in 15% of the analyzed patients no T cell frequencies could be detected because they had insufficient numbers of T cells, APCs, or had a too high background of CD154 expression. These limitations are most likely associated with the previous treatment and probably transient. Furthermore, in most of these cases just a single measurement could be performed. Therefore, repeated measurements and adjusted sample volumes might further reduce the number of drop out. Nevertheless, the induction of CD154 expression requires processing of the fungal lysates by functional APCs and the background expression of CD154 has to be sufficiently low to detect an increase of fungus-reactive T cells. Such limitations could be overcome by measuring antigen-specific T cells with peptide-MHC class II multimers that directly bind to the T cell receptor, independent of the activation status of the T cells. However, as already stated above, the usage of multimers requires prior knowledge of exactly defined antigenic epitopes and needs to be matched with the individual MHC haplotypes of the patients. Since no immuno-dominant fungal antigens are known so far, multimer-detection is not applicable to analyze fungus-specific T cells. Indeed, the analysis of T cell target proteins from *A. fumigatus* revealed a broad and heterogeneous reactivity to a variety of different proteins in healthy donors, questioning the existence of one or few immuno-dominant fungal antigens (Bacher et al., manuscript 6, *submitted*). In addition, the prevalent protein-specificity of the specific T cells may differ between individual patients. Therefore the possibility to use complete lysates from different fungal species, which are naturally processed by the APCs, enables to cover the broad repertoire of fungal antigens, independent of host-specific factors (*e.g.* MHC restriction and/ or TCR repertoire specificity).

Besides the reliable detection of IFI, it is essential to know which fungal pathogen causes the invasive disease, since different fungal pathogens require adjusted anti-fungal treatment (Cornely et al., 2009; Kuse et al., 2007; van Burik et al., 2006; Vehreschild et al., 2013). However, even if the fungus has been isolated from biopsies or lung resection, the microscopic discrimination of similar filamentous fungi is quite challenging (Ostrosky-Zeichner, 2012). The measurement of fungus-specific $CD4^+$ T cells allowed a clear distinction between the most frequent invasive filamentous fungal genera *Aspergillus* spp. and Mucorales (Bacher et al., manuscript 5, *in preparation*). This was confirmed by the re-stimulation of expanded fungus-reactive T cell lines, which showed only low T cell cross-reactivity between *Aspergillus* and Mucorales species. However, within different *Aspergillus* species, a strong T cell cross-reactivity was observed, indicating that discrimination on a species level might be

difficult or requires comparative testing against various *A. fumigatus* species. In addition, cross-reactivity was observed within *Scedosporia* spp. and *Fusarium* spp. specific T cell lines. This demonstrates, that depending on the fungal pathogen, cross-reactive T cells may account for different proportions of the total fungal species-reactive T cell pool and that discrimination of some fungal pathogens requires more detailed analysis. This has to be considered regarding the diagnosis of IFI, based on specific T cell frequency alterations in high-risk patients.

By comparing the results of the $CD154^+$ T cell assay with the current clinical diagnosis, the test yielded a high sensitivity and specificity in the analyzed patients cohort of 91.7% and 92.3%, respectively. For comparison, other non-invasive blood and BAL based standard diagnostic assays reported sensitivities and specificities for ELISA measurement of cell wall β-1,3-D-glucan of 50% and 99% (Lamoth et al., 2012), for galactomannan of 78% and 81% (Leeflang et al., 2008) and for PCR methods of 75% and 87% (Mengoli et al., 2009). In addition, galactomannan, the test with the highest sensitivity, is restricted to the detection of aspergillosis (De Pauw et al., 2008). Thus the results obtained with the T cell assay in the analyzed patients cohort clearly exceeded the results of currently available blood tests for invasive fungal infections. Furthermore, the comparison of the T cell assay with the clinical diagnosis was performed by considering patients with proven and probable IFI as having confirmed IFI, whereas those patients with possible and no IFI were handled as having no IFI. Thus, the values for sensitivity and specificity of the T cell assay may even be higher, since the reference diagnosis is ambiguous in cases of probable and possible IFI. Indeed, regarding only healthy donors and proven IFI, the test yielded a sensitivity and sensitivity of each 100%, since no donor in the healthy control group (n = 100) had increased T cell frequencies and all six cases of proven IFI were identified by increased T cell signals. Of course, these results have to be evaluated in statistically adequate patient cohorts.

The presence of high frequencies of fungus-specific $CD4^+$ T cells in IFI patients also raises an important immunological question. For instance, despite the strong expansion of fungus-specific $CD4^+$ T cells, the fungal infection is not effectively cleared by the patients´ immune system. Besides their presence in peripheral blood, increased frequencies of fungus-specific $CD4^+$ T cells could also be detected in lung resections from infected patients (Bacher and Scheffold, unpublished data), indicating that the specific T cells are indeed present at the site of infection. However, irrespective of increased frequencies of fungus-reactive T cells, the absolute numbers of $CD4^+$ T cells in peripheral blood were strongly reduced in most patients.

Therefore, despite markedly increased frequencies in IFI patients, the absolute numbers of fungus-specific T cells did not exceed the range of healthy donors for most patients or time points (Bacher et al., manuscript 5, *in preparation*). Thus one can hypothesize, that expansion or persistence of fungus-specific T cells in this strongly immune-suppressed patients cohort is only insufficient. This would also be in line with the fact that the fungus-specific T cells revealed only limited cytokine producing capacity, indicating only restricted effector functions of fungus-reactive T cells during fungal infections (Bacher et al., manuscript 5, *in preparation*).

In summary, these results may in part explain why invasive mold infections are not effectively cleared, despite the activation of a fungus-specific $CD4^+$ T cell response and give a further rational for adoptive T cell transfer approaches to reconstitute and/or assist the anti-fungal $CD4^+$ T cell response in IFI patients. The multi-parameter characterization of single *A. fumigatus* protein-specific T cell responses provided no evidence for the existence of one or a few immuno-dominant antigens but revealed a quite heterogeneous T cell response to a variety of different antigens in healthy donors. Nevertheless, it enabled the classification of various proteins into "immunogenic", "tolerogenic" or "exhausting" groups (Bacher et al., manuscript 6, *submitted*). Thus, the pre-selection of certain antigens according to the phenotypic and functional characteristics of the reactive T cells might help to define immuno-stimulatory and protective targets for vaccination strategies or adoptive T cell therapy approaches and could even be applied to define a selected set of target antigens for individual patients. Since in healthy donors the *A. fumigatus*-specific T cell response is strongly counter-regulated by specific Treg cells (Bacher et al., 2013; manuscript 3), it will be important to determine a potential role for Treg as additional risk factor for preventing anti-fungal immunity in immunocompromised patients. However, also other parameters such as presence and function of innate immune cells to execute T cell signals might be causative for the failure to resolve the fungal infections in IFI patients and remain to be explored.

5 Summary

Invasive fungal infections are a major cause of infection related mortality in immunocompromised patients. Furthermore, inappropriate immune responses to fungal pathogens are associated with disease exacerbations in particular in patients with allergies or compromised lung functions, such as asthma or cystic fibrosis. Antigen-specific $CD4^+$ T cells have been suggested to play a major role in anti-fungal immune responses. However, the exact quantification and detailed characterization of fungus-specific $CD4^+$ T cells in humans has been hampered so far by the lack of appropriate methods for their identification. To overcome this technology gap, a flow-cytometric method was developed that allows for the first time the direct *ex vivo* detection and comprehensive characterization of the entire fungus-reactive $CD4^+$ T cell compartment from human peripheral blood, *i.e.* conventional memory and naive $CD4^+$ T cells (Tcon), as well as regulatory T cells (Treg). Based on the magnetic pre-enrichment of fungal antigen stimulated $CD154^+$ Tcon and $CD137^+$ Treg, fungus-reactive $CD4^+$ T cells can be analyzed from large starting cell numbers, which allows the collection of sufficient numbers of target cells for subsequent phenotypic and functional analyses with high statistical precision. This technology was used to perform a detailed characterization of the $CD4^+$ T cell response against two of the major fungal pathogens in humans, the constantly inhaled airborne fungus *Aspergillus fumigatus* and the gut- and skin-residing commensal *Candida albicans*.

The analysis of *A. fumigatus* and *C. albicans*-reactive $CD4^+$ T cells revealed profound differences in the T cell response against both fungi, suggesting different pathogen-related and/or tissue compartment-specific immune mechanisms. Interestingly, both, specific Tcon and Treg, could readily be detected in the blood of all healthy adult donors, reflecting most likely the frequently encounter with both fungal pathogens. However, whereas *A. fumigatus* generates predominantly a memory Treg response and a large part of the specific Tcon repertoire was still in a naive state, a clear dominating memory Tcon response could be detected against *C. albicans* with only minor contribution of Treg. This tolerance pattern of the *A. fumigatus*-specific T cell response was abrogated in patients with severe *A. fumigatus* allergies, suggesting that counter-regulation of anti-*A. fumigatus* immune responses by Treg might be an important mechanism for prevention of allergic disorders.

The further functional characterization of fungus-specific Tcon revealed a strong expansion of *C. albicans*-specific Th17 cells, whereas against *A. fumigatus*, as well as other airborne fungi, Th17 cells were only rare, questioning the current paradigm that Th17 cells are in general mandatory in anti-fungal immune defense. Importantly, in all analyzed donors, the *A. fumigatus*-reactive memory T cell repertoire always contained a significant population of cells with cross-reactivity to *C. albicans* antigens and only these cross-reactive cells displayed phenotypical characteristics of Th17 cells and were able to produce IL-17 and IL-22. This finding might reflect a general mechanism, how the intestinal microbiota can influence local as well as systemic immune homeostasis and pathology by specific expansion of particular Th cell subsets with reactivity to gastrointestinal and gut-distal antigens.

Importantly, the possibility to characterize in detail the anti-fungal T cell response in healthy donors offered the opportunity to find disease-associated changes in relevant patient groups. The analysis of a cohort of immunocompromised hematologic patients revealed that patients with invasive fungal infections displayed strongly increased frequencies of fungus-reactive T cells, compared to healthy controls or patients at risk, but without fungal infections. These data strongly suggest that the measurement of antigen-specific T cell responses in high-risk patients might be a potential parameter for the diagnosis of invasive fungal infections from peripheral blood samples. Despite increased frequencies within the $CD4^+$ T cell compartment, the absolute numbers of fungus-reactive T cells were not augmented and effector functions were only limited. Hence, adoptive transfer approaches of fungus-specific T cells might confer protective immunity by reconstituting the anti-fungal $CD4^+$ T cell response in high-risk patients, but are mainly hampered by the lacking knowledge about protective T cell targets.

Indeed, the analysis of various *A. fumigatus* development stages, subcellular fractions and single *A. fumigatus* proteins indicated that the T cell response is highly divers, questioning the existence of one or few immuno-dominant target antigens. However, the in depth characterization of T cells specific for single *A. fumigatus* proteins revealed quantitative and qualitative differences which allowed to classify the various proteins into "immunogenic" "exhausting" or irrelevant "non-target" groups. Thus this approach may provide a general screening procedure to identify appropriate T cell targets for adoptive T cell therapy or vaccine development enabling pre-emptive or therapeutic intervention against the devastating fungal infections in immunocompromised patients.

6 Zusammenfassung

Invasive Pilzinfektionen stellen eine Hauptursache für die Mortalität bei immunsupprimierten Patienten dar. Weiterhin sind unangemessene Immunreaktionen gegen Pilze mit Krankheits-Verschlechterung in Patienten mit Allergie oder beeinträchtigter Lungenfunktion, z.B. bei Asthma oder Mukoviszidose, assoziiert. Verschiedene Daten deuten darauf hin, dass Antigen-spezifische $CD4^+$ T Zellen eine wesentliche Rolle in der Immunantwort gegen pathogene Pilze spielen. Allerdings konnte eine exakte Quantifizierung und detaillierte Charakterisierung von Pilz-spezifischen $CD4^+$ T Zellen im Menschen bislang nicht durchgeführt werden, da keine geeigneten Methoden für deren Identifizierung zur Verfügung standen. Um diese Technologie-Lücke zu schließen, wurde eine durchflusszytometrische Methode entwickelt, die es erstmals ermöglicht, das gesamte Pilz-spezifische $CD4^+$ T Zell Repertoire, d.h. konventionelle Gedächtnis- und Naive T Zellen (Tcon), sowie regulatorische T Zellen (Treg), direkt *ex vivo* im menschlichen peripheren Blut zu detektieren und umfassend zu charakterisieren. Basierend auf der magnetischen Voranreicherung von Antigen-stimulierten $CD154^+$ Tcon und $CD137^+$ Treg, können Pilz-spezifische $CD4^+$ T Zellen aus großen Start-Zellzahlen analysiert werden. Dies ermöglicht es, ausreichende Mengen an Zielzellen zu detektieren, um anschließende phänotypische und funktionelle Analysen mit hoher statistischer Präzision durchzuführen. Diese Technologie wurde angewandt, um die menschliche $CD4^+$ T Zell Antwort gegen zwei bedeutende human-pathogene Pilze, den dauerhaft inhalierten Schimmelpilz *Aspergillus fumigatus*, sowie den Darm- und Haut-besiedelnden Hefepilz *Candida albicans,* detailliert zu charakterisieren.

Die Analyse von *A. fumigatus*- und *C. albicans*-reaktiven $CD4^+$ T Zellen zeigte umfassende Unterschiede in der T Zell Antwort gegen beide Pilze auf. Dies lässt auf verschiedene Pathogen-bezogene und/ oder Gewebekompartiment-spezifische Immunmechanismen schließen. Interessanterweise konnten sowohl spezifische Tcon als auch Treg gegen beide Pilze im Blut von erwachsenen Menschen nachgewiesen werden, was höchstwahrscheinlich den regelmäßigen Kontakt des Immunsystems mit beiden Pilzen reflektiert. Während *A. fumigatus* jedoch überwiegend eine Gedächtnis Treg Antwort generiert und ein Großteil der spezifischen Tcon Antwort noch einen naiven Phänotyp aufweist, ist die T Zell Antwort gegen *C. albicans* eindeutig durch konventionelle Gedächtnis T Zellen dominiert, mit nur geringer Beteiligung von Treg. Dieses Toleranz-Muster der *A. fumigatus*-spezifischen T Zell Antwort ist in Patienten mit schwerer *Aspergillus* Allergie aufgehoben, was vermuten lässt, dass die Regulation der *A. fumigatus*-spezifischen T Zell Antwort durch Treg einen wichtigen Mechanismus darstellt, um allergische Immunreaktionen zu unterdrücken.

Die weitere Charakterisierung der Pilz-spezifischen Tcon zeigte eine starke Expansion von *C. albicans*-spezifischen Th17 Zellen, während sowohl gegen *A. fumigatus*, als auch andere luftübertragene Pilze, Th17 Zellen unterrepräsentiert waren. Dies stellt das derzeitige Paradigma in Frage, dass Th17 Zellen in anti-Pilz Immunantworten generell zwingend erforderlich sind. Weiterhin konnte gezeigt werden, dass das *A. fumigatus*-spezifische Gedächtnis T Zell Repertoire eine signifikante Population von Zellen enthält, welche kreuzreaktiv mit *C. albicans* Antigenen sind und dass nur diese kreuzreaktiven Zellen phänotypische Marker von Th17 Zellen exprimieren und in der Lage sind, die Th17 Zytokine IL-17 und IL-22 zu sekretieren. Diese gezielte Induktion einer T Zell Sub-Population durch Kreuzreaktivität zwischen gastro-intestinalen und peripheren Antigenen könnte einen generellen Mechanismus darstellen, wie die intestinale Mikrobiota sowohl lokale, als auch systemische Immunhomöostase und Pathologie beeinflussen kann.

Die Möglichkeit die anti-Pilz T Zell Antwort in gesunden Spendern detailliert zu charakterisieren stellt eine Grundvoraussetzung dar, um krankheitsassoziierte Änderungen in relevanten Patientengruppen zu erkennen. Die Analyse von immunsupprimierten hämatologischen Patienten zeigte, dass im Blut von pilzinfizierten Patienten stark erhöhte Frequenzen an Pilz-reaktiven T Zellen detektiert werden können, im Gegensatz zu gesunden Menschen oder Risikopatienten ohne Pilzinfektion. Daher scheint die Messung von Antigen-spezifischen T Zell Antworten in Risikopatienten ein vielversprechender Parameter für die Diagnose von invasiven Pilzinfektionen zu sein. Trotz erhöhter Frequenzen innerhalb des $CD4^+$ T Zell Kompartiments, war die absolute Zahl an Pilz-spezifischen $CD4^+$ T Zellen jedoch nicht erhöht und die T Zell Effektor Funktionen nur limitiert. Daher wären adoptive Transferverfahren von Pilz-spezifischen T Zellen denkbar, um die anti-Pilz T Zell Antwort in Risikopatienten wieder herzustellen. Ein solcher Ansatz wird hauptsächlich dadurch behindert, dass keine protektiven T Zell Zielantigene von Pilzen bekannt sind.

Die Analyse von Antigenen verschiedener Morphotypen, zellulärer Proteinfraktionen und Einzelproteine zeigte in der Tat, dass die T Zell Antwort gegen *A. fumigatus* sehr divers ist, was die Existenz von einem oder wenigen immun-dominanten Zielantigenen in Frage stellt. Die detaillierte Charakterisierung der T Zell Antwort gegen einzelne *A. fumigatus* Proteine zeigte jedoch quantitative und qualitative Unterschiede auf, die eine Klassifizierung der Proteine in „immunogene“, „ausbrennende“ oder irrelevante „Nicht-Ziel“ Antigene ermöglichte. Dieser Ansatz könnte eine generelle Screening Methode darstellen, um geeignete T Zell Antigene für adoptive T Zell Therapien oder Vakzinierung zu identifizieren und so präventive oder therapeutische Möglichkeiten gegen die verheerenden Pilzinfektionen in immunsupprimierten Patienten zu entwickeln.

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